Organic carbon uptake by bacteria with different live strategies – Successions and unexplored food web interactions

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Ester Eckert

von Bern BE

Promotionskomitee
Prof. Dr. Jakob Pernthaler (Leitung der Dissertation, Vorsitz)
Prof. Dr. Leo Eberl
PD Dr. Martin Hahn

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Diese Arbeit ist meiner Grossmutter, Alice Eckert, gewidmet.

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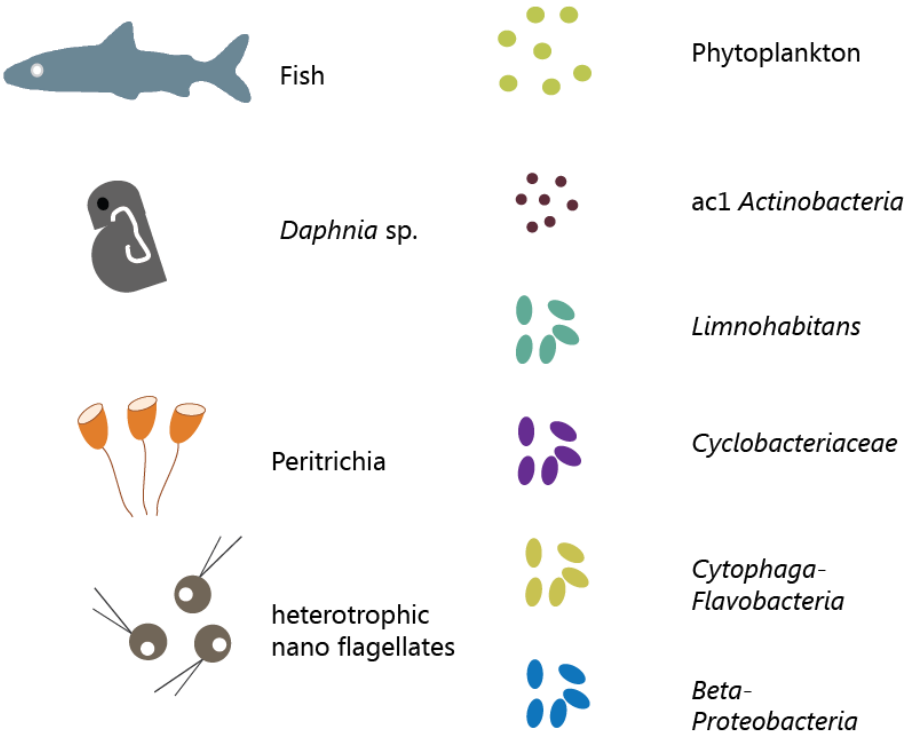
GLOSSARY

Abbreviations

ac1	ac1 of *Actinobacteria*
Beta	Beta-*Proteobacteria*
CF	*Cytophaga-Flavobacteria*
Chl a	Chlorophyll *a*
(D)OC	(dissolved) organic carbon
FISH	Fluorescence *in situ* hybridisation
HNF	heterotrophic nano-flagellates
LD2	Bacteria affiliated with *Candidatus* Aquirestis calciphila
Lhb	*Limnohabitans* cluster of Beta
NAG	N-acetyl-glucosamine

Pictograms of organisms used in schematic figures



SUMMARY

SPRING IN TEMPERATE LAKES, SUCH AS LAKE ZURICH, IS DEFINED BY ASYNCHRONOUS BLOOMS OF VARIOUS MICROBIAL POPULATIONS. FOLLOWING A STEEP INCREASE OF BIOMASS OF PHYTOPLANKTON, FAST-GROWING BACTERIA RISE IN ABUNDANCE, SINCE THEY PROFIT FROM THE ORGANIC CARBON EXUDATION OF THE PRIMARY PORDUCERS. SUCH BACTERIA ARE AFFILIATED WITH *CYTOPHAGA-FLAVOBACTERIA*, NAMELY *FLUVIICOLA* SP. OR *CYCLOBACTERIACEAE*, OR WITH THE *LIMNOHABITANS* CLUSTER OF BETA-*PROTEOBACTERIA*. THESE POPULATIONS ARE SUBSEQUENTLY DIMINISHED BY FEEDING OF HETEROTROPHIC NANO-FLAGELLATES, OPENING A NICHE FOR GRAZING-RESISTANT BACTERIA SUCH AS AC1 *ACTINOBACTERIA* AND FILAMENTOUS LD2 BACTERIA.

THESE PHYLOTYPES DO NOT ONLY PROFIT FROM THEIR GRAZING-RESISTANT MORPHOLOGY, BUT ALSO SEEM TO BE SPECIALISTS FOR THE DEGRADATION OF SUBSTRATES RELEASED BY FLAGELLATE GRAZING, SUCH AS THE BACTERIAL CELL WALL COMPONENT N-ACETYL-GLUCOSAMINE (NAG). DURING THE SPRING PHYTOPLANKTON BLOOM 2009 IN LAKE ZURICH THE UPTAKE OF NAG WAS FIRST DOMINATED BY FAST-GROWING BACTERIA, WHILE GRAZING-RESISTANT BACTERIA LATER CONSTITUTED THE GREATER PERCENTAGE OF NAG-ACTIVE CELLS. MOREOVER, DESPITE BEING FREE-LIVING, THEY WERE HIGHLY ENRICHED ON POLYMERS SUCH AS PEPTIDOGLYCAN AND CHITIN, PRESUMABLY BECAUSE THE SUBSTRATES WERE PRE-DIGESTED BY FLAGELLATES. THUS, SUBSTRATES SUCH AS NAG ARE PASSED ON DIFFERENT ROUTES THROUGH THE FOOD WEB: WHEN INCORPORATED BY FAST-GROWING BACTERIA THEY ARE FURTHER TAKEN UP BY HETEROTROPHIC NANO-FLAGELLATES THAT ARE EATEN BY *DAPHNIA* SPP. IF, HOWEVER, TAKEN UP BY AC1 *ACTINOBACTERIA* THE ORGANIC CARBON FROM THESES SUBSTRATES MAY NEVER REACH ORGANISMS OF HIGHER TROPHIC LEVELS, SINCE AC1 ARE PRESUMABLY SUBJECTED TO VIRAL LYSIS.

AFTER PHYTOPLANKTON BLOOMS ZOOPLANKTON SPECIES SUCH AS THE GENUS *DAPHNIA* USUALLY CONSTITUTE MAJOR POPULATIONS IN TEMPERATE LAKES. DAPHNIDS ARE COMMONLY ASSOCIATED WITH HETEROTROPHIC BACTERIA THAT LIVE ON THEIR FILTER COMBS AND PROFIT FROM THE FILTRATION ACTIVITY OF THE ANIMALS. MANY OF THESE BACTERIA ARE AFFILIATED WITH *LIMNOHABITANS* SP. AND ARE HIGHLY ACTIVE IN THE INCORPORATION OF DISSOLVED SUBSTRATES SUCH AS THE AMINO ACID LEUCINE. IN LAKE ZURICH UP TO 10% OF THE LEUCINE UPTAKE WAS CONDUCTED BY BACTERIA ATTACHED TO DAPHNIDS. THIS HAS IMPLICATIONS FOR THE FOOD WEB, SINCE THE EPIBIONTS ARE, TOGETHER WITH THEIR HOST, CONSUMED BY FISH. THUS THE UPTAKE OF DISSOLVED ORGANIC CARBON BY EPIBIONTS PROVIDES A SHORT-CUT THROUGH THE FOOD WEB, WHERE BACTERIALLY INCORPORATED CARBON IS DIRECTLY PASSED ON TO FISH.

THE RELEASE OF ORGANIC CARBON BY FLAGELLATES AND THE UPTAKE BY GRAZING-RESISTANT BACTERIA AS WELL AS THE UPTAKE OF CARBON BY *DAPHNIA* SPP. EPIBIONTS ARE BOTH EXAMPLES FOR HIDDEN FOOD WEB INTERACTIONS INVOLVING MICROBES. ELUCIDATION OF SUCH INTERACTIONS AND THEIR INCORPORATION INTO FRESHWATER FOOD WEB ANALYSIS MAY SIGNIFICANTLY ALTER OUR UNDERSTANDING OF SUCH SYSTEMS.

ZUSAMMENFASSUNG

DAS FRÜHJAHR VON SEEN IN GEMÄSSIGTEN BREITEN, WIE Z.B. DEM ZÜRICHSEE, ZEICHNET SICH DURCH EINE SUKZESSION VERSCHIEDENER BLÜTEN VON MIKROBIELLEN POPULATIONEN AUS. DEM STEILEN ANSTIEG DER BIOMASSE VON EINZELLIGEM PHYTOPLANKTON, FOLGEN HOHE ABUNDANZEN SCHNELL-WACHSENDER BAKTERIEN. DIESE BAKTERIEN LASSEN SICH TAXONOMISCH ZUM BEISPIEL FOLGENDEN EINHEITEN ZUORDNEN: *FLUVIICOLA* SP. ODER *CYCLOBACTERIACEAE* DER *CYTOPHAGA-FLAVOBACTEIRA* ODER DER GATTUNG *LIMNOHABITANS* (BETA-*PROTEOBACTERIA*). DIE BAKTERIEN-POPULATIONEN WERDEN IN FOLGE DURCH DIE FRASSAKTIVITÄT VON HETEROTROPHEN NANOFLAGELLATEN DEZIMIERT. DIES ÖFFNET EINE NEUE NISCHE FÜR FRASS-RESISTENTE BAKTERIEN WIE AC1 *ACTINOBACTERIA* UND FÄDIGEN LD2 BAKTERIEN.

DIESE PHYLOGENETISCHEN GRUPPEN PROFITIEREN NICHT NUR VON IHRER FRASS-RESISTENTEN MORPHOLOGIE SONDERN SCHEINEN AUCH AUF SUBSTRATE SPEZIALISIERT ZU SEIN DIE BEIM FRASSVERHALTEN DER FLAGELLATEN FREIGESETZT WERDEN. DIESE SUBSTRATE BEINHALTEN ZUM BEISPIEL DIE BAKTERIELLE ZELLWANDUNTEREINHEIT N-ACETYL-GLUCOSAMIN. WÄHREND SCHNELL-WACHSENDE BAKTERIEN DIE AUFNAHME DIESES SUBSTRATES AM ANFANG DER BLÜTE DOMINIERTEN, ZEIGTEN DANACH FRASS-RESISTENTE BAKTERIEN EINE EFFIZIENTERE ASSIMILATION. DARÜBER HINAUS WAREN AC1 UND LD2 FILAMENTE ERFOLGREICH AUF POLYMEREN WIE PEPTIDOGLYCAN UND CHITIN ANREICHERBAR, OBWOHL ES SICH EIGENTLICH UM REIN PLANKTISCHE BAKTERIENGRUPPEN HANDELT. WAHRSCHEINLICH WAR DIES DURCH EINEN „VORVERDAUUNG" DER SUBSTRATE DURCH DIE FLAGELLATEN MÖGLICH.

DIE FRÜHJAHRESBLÜTE WIRD GEWÖHNLICH DURCH DEN FRASS VON METAZOOPLANKTERN, WIE Z.B. DER GATTUNG *DAPHNIA*, BEENDET. VIELE DIESER DAPHNIEN SIND MIT HETEROTROPHEN BAKTERIEN VERGESELLSCHAFTET, WOBEI DIE MIKROORGANISMEN INSBESONDERS DIE FILTRATIONSKÄMME DER DAPHNIEN BESIEDELN. DORT PROFITIEREN SIE VON DER FILTRATIONSAKTIVITÄT DES KLEINKREBSES, WODUCRH EINE STÄNDIGE ZUFUHR VON FRISCHWASSER GARANTIERT WIRD. FOLGLICH SIND DIESE AUFWUCHS-BAKTERIEN SEHR EFFIZIENT IN DER AUFNAHME VON ORGANISCHEM KOHLENSTOFF WIE ZUM BEISPIEL DER AMINOSÄURE LEUCIN. IN WASSERPROBEN AUS DEM ZÜRICHSEE WURDEN BIS ZU 10% DER LEUCINAUFNAHME DURCH BAKTERIEN AUF DAPHNIEN VERURSACHT. DIESE BEOBACHTUNG HAT KONSEQUENZEN FÜR DAS GESAMTE NAHRUNGSNETZ, DA DIE BAKTERIELLEN EPIBIONTEN ZUSAMMEN MIT IHREM WIRT VON PLANKTIVOREN FISCHEN GEFRESSEN WERDEN. SOMIT BILDET DIE AUFNAHME VON ORGANISCHEM KOHLENSTOFF DURCH EPIBIONTEN EINE „ABKÜRZUNG" INNERHALB DES NAHRUNGSNETZES, BEI DER TROPHISCHE EBENEN ÜBERSPRUNGEN WERDEN, UND DER BAKTERIELL AUFGENOMMENE ORGANISCHE KOHLENSTOFF DIREKTER ZU FISCHEN GELANGT.

DIE FREISETZUNG VON ORGANISCHEM KOHLENSTOFF DURCH FLAGELLATEN UND DEREN AUFNAHME DURCH FRASS-RESISTENTE BAKTERIEN, WIE AUCH DIE AUFNAHME VON KOHLENSTOFF DURCH *DAPHNIA*-EPIBIONTEN SIND BEISPIELE FÜR OFT VERNACHLÄSSIGTE INTERAKTIONEN IN NAHRUNGSNETZEN. DIE ERFORSCHUNG DIESER WEITGEHEND UNBEKANNTEN PROZESSE UND DEREN BERÜCKSICHTIGUNG IN NAHRUNGSNETZANALYSEN VON BINNENGEWÄSSERN WIRD UNSER VERSTÄNDNIS FÜR DIESE SYSTEME NACHHALTIG VERBESSERN.

PREFACE

This dissertation comprises three studies, summarised in three manuscripts, of which two were published in *Environmental Microbiology* (Eckert et al, 2012 & 2013) and one is currently submitted. The first article is a descriptive study of the development of various bacterial populations, their uptake of leucine and N-acetyl-glucosamine, and other biotic and abiotic factors during a phytoplankton spring bloom (*Article I*). From there we formulated a hypothesis that was tested in the second study, namely that grazing-resistant bacterial populations such as ac1 *Actinobacteria* and filamentous bacteria profit from substrates released through flagellate grazing (*Article II)*. In the third study we further followed specific compounds and evaluated their uptake by epibiotic bacteria on crustacean *Daphnia* spp. (*Article III)*.

Article I: Eckert, E.M., Salcher, M.M., Posch, T., Eugster, B., and Pernthaler, J. (2012) Rapid successions affect microbial N-acetyl-glucosamine uptake patterns during a lacustrine spring phytoplankton bloom. *Environ Microbiol* 14: 794-806.

Article II: Eckert, E.M., Baumgartner, M., Huber, I.M., and Pernthaler, J. (2013) Grazing resistant freshwater bacteria profit from chitin and cell-wall-derived organic carbon. *Environ Microbiol* 15: 2019-2030.

Article III: Eckert, E.M., and Pernthaler, J. (submitted) Bacterial epibionts of Daphnia are a potential route for the efficient transfer of dissolved organic carbon within freshwater food webs.

In the following pages I describe how conclusions for these studies were drawn and on which basis new hypotheses were formulated. I shall support my statements with examples from both the literature, and the three above mentioned studies and I shall also include some unpublished data. Moreover, I will try to put all the core findings of the studies in a food web context. To exemplify some of these mechanisms I intend to concentrate on few populations of bacteria, protozoa and the zooplankton genus *Daphnia*. It is not my intention to summarize all aspects of my work in this section since they are, to my opinion, already sufficiently formulated in the three manuscripts. I shall rather reflect my work of the last years and try to carve out some implications and perspectives for further studies. Thus, the text is not organised in introduction and discussion but in chapters that relate to some aspects of the studies.

1 TRANSLATING SUCCESSIONS INTO FOOD WEB HYPOTHESIS

Lake Zurich is a subalpine oligo-mesotrophic lake (Bossard*, et al.*, 2001). Freshwater food webs of temperate regions are highly dynamic, due to the impact of the seasons on the epilimnetic temperature[1] (Simon*, et al.*, 1998). Initiated by the steep increase of phytoplankton biomass in early spring, a variety of organismic groups fluctuate in abundance throughout the period of the so-called spring bloom. Unicellular primary producers exude organic carbon (OC), which serves as a substrate for the carbon limited heterotrophic bacteria (Schweitzer & Simon, 1995, Felip*, et al.*, 1996). In turn, the increasing numbers of bacteria promote the growth of heterotrophic nano-flagellates (HNF) which feed on the bacterial assemblage (Weisse, 1991) and provide a niche for alternative bacterial groups, not affected by grazing. Generally phytoplankton blooms are followed by high abundances of

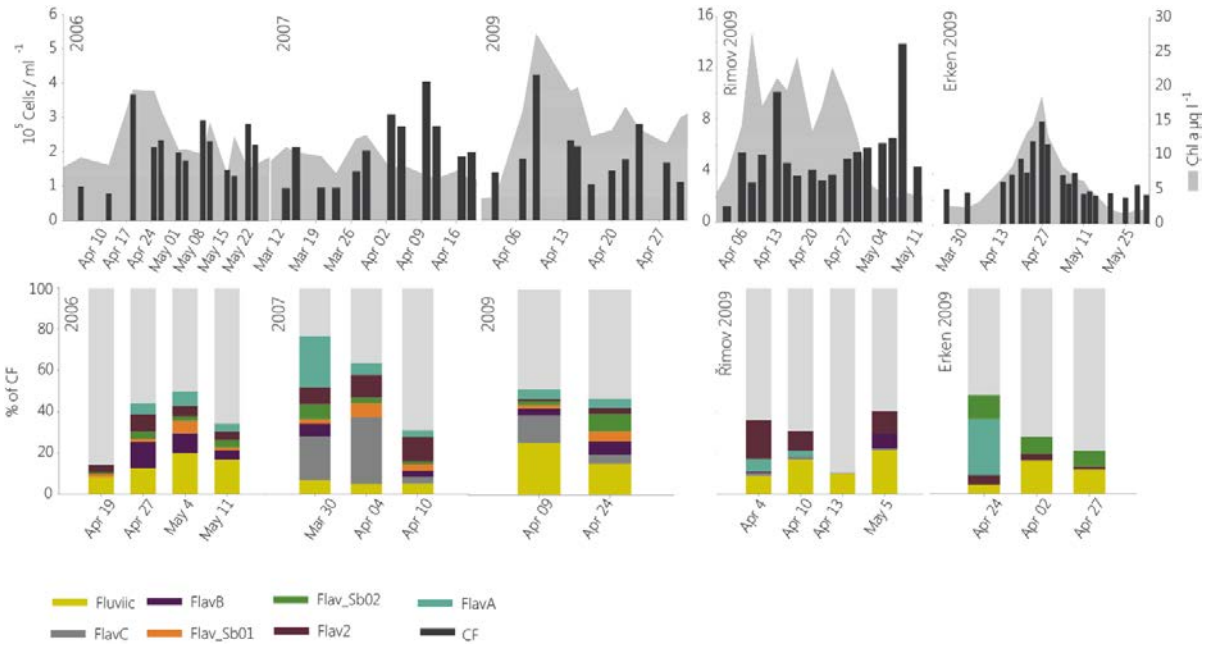


Fig 1 *Flavobacteria* populations in Lake Zurich during Spring 2006, 2007 & 2009, and in the Řimov Reservoir (Czech Republic) and Lake Erken (Sweden) in 2009. Upper panels: Development of chlorophyll *a* (Chl a) and all *Flavobacteria* hybridised with the FISH probe CF319a (CF). Lower panels: Contributions of *Flavobacteria* subclusters at the respective CF319a maximum abundances. Probes: CF319a (Manz*, et al.*, 1996), Fluviicola (Fluviic,(Salcher*, et al.*, 2011)), Flav2 (Zeder*, et al.*, 2009) FlavA, FlavB and FlavC (Neuenschwander et al, submitted), FlavSb01 & FlavSb02 (unpublished).

[1] The articles of this PhD thesis, as the majority of literature on lakes, is based on studies of the pelagial of the epilimnion of oligo-mesotrophic lakes, thus, if not specified otherwise this is the region I refer to when speaking about the lake.

zooplankton species (Simon*, et al.*, 1998), in Lake Zurich usually dominated by daphnids of the *Daphnia longispina*-complex.

Despite the pronounced dynamics of these microbial blooms, synchrony and repeated seasonality is commonly observed (Sommer, 1986, Kent*, et al.*, 2007, Rusak*, et al.*, 2009) . In other words; patterns observed in a given lake at a given time, are often paralleled in similar, physically separated lakes, and repeated in the following years, provided that the weather conditions are similar. This does not necessarily include all occurring bacterial phylotypes; it seems that some groups are more regularly observed, while another part of the microbial assemblage is more variable (Boucher*, et al.*, 2006). For example; we compared the abundance of seven vernal populations of *Cytophaga-Flavobacteria* (CF) over three years in Lake Zurich, Lake Erken (Sweden) and a freshwater reservoir (Římov Reservoir, Czech Republic)[2] (Fig 1): Some populations were abundant in all spring samples such as *Fluviicola* related bacteria or Flav2 whereas others were only found on some dates and samples (e.g. FlavA and FlavC) (Fig 1, unpublished data). However, all of the analysed groups were at least found in two years in Lake Zurich and in one of the other two systems. Thus, the success of microbial taxa during the spring season is not random, but rather comprises organisms that are adapted to a temporary short-lived niche space. Microbial successions then derive from species that differ in their biological role and find ideal growth conditions at different times in the fluctuating environment (Chesson & Huntly, 1997).

The two main factors defining the niches for aquatic bacteria are the availability of organic and inorganic nutrients and agents of mortality such as viruses and HNF (Thingstad & Lignell, 1997). Throughout the last two decades many experimental studies have determined how *bottom-up* (nutrients) and *top-down* (predation) control factors influence bacterial growth as well as genotypic and phenotypic succession (e.g. Jürgens*, et al.*, 1999, Hahn & Höfle, 2001, Salcher*, et al.*, 2007, Corno & Jürgens, 2008). It has early been proposed that aquatic bacteria may be classified in two main adaptive guilds: fast-growing and grazing-resistant bacteria (e.g. Weinbauer & Höfle, 1998). Some of the fast-growing or opportunistically-growing bacteria are free-living and others attach to particles, where OC concentrations and turnover are typically high (Simon*, et al.*, 2002, Allgaier & Grossart, 2006, Grossart*, et al.*, 2007). In some cases the microbes may also attach and detach from such organic flocs (Grossart*, et al.*, 2003). Members of the opportunistically growing bacterial guild were observed to enrich

[2] Samples were kindly collected and provided by Stefan Bertilsson and Karel Šimek and their respective working groups.

rapidly as soon as grazers were removed from the system or when nutrient concentrations were increased (e.g. Šimek*, et al.*, 2003, Salcher*, et al.*, 2007, Zeder*, et al.*, 2009) and high HNF grazing was even observed on bacteria attached to aggregates (Kiørboe*, et al.*, 2004, Corno*, et al.*, 2012). Thus, fast-growing bacteria seem to have developed an efficient substrate uptake machinery, are, however, highly vulnerable to protistan grazing. One example for bacteria that follow such growth patterns and are common in lakes are related to *Limnohabitans* spp. of *Beta-Proteobacteria* (Beta) and targeted by the probe R-Bt065 (Lhb) (Šimek*, et al.*, 2005). In culture Lhb bacteria seem to profit from the presence of algae, particularly cryptophytes that are known for pronounced organic carbon exudation (Šimek*, et al.*, 2011). These bacteria were highly enriched in an experimental treatment when the water was prefiltered over 5 µm to remove protistan grazers (Šimek*, et al.*, 2005) (Fig 2A). Moreover taxon specific hybridisations could localise many of them in food vacuoles of HNF (Jezbera*, et al.*, 2005).

At the other end of the spectrum grazing-resistant bacteria enrich when grazing-pressure on the community is increased (Pernthaler, 2005). Part of the lake bacteria are grazing protected due to morphological and structural features, meaning that very small cells (*ultramicrobacteria*) with rigid cell walls and long filamentous cells are less commonly ingested by HNF (Langenheder & Jürgens, 2001, Tarao*, et al.*, 2009). The most prominent grazing-resistant bacteria during spring time in lakes belong to the ac1 tribe of *Actinobacteria* (ac1) (Glöckner*, et al.*, 2000, Allgaier & Grossart, 2006, Salcher*, et al.*, 2010). These bacteria only enriched in experiments under grazing pressure, i.e. when other bacteria were eliminated from the community (Pernthaler*, et al.*, 2001) (Fig 2A). Moreover in a co-culture with other very small cells ac1 bacteria were still consumed at a lower frequency, presumably due to their gram-positive cell wall structure and other cell envelope properties (Tarao*, et al.*, 2009). Examples for filamentous bacteria are found in two dominant groups affiliated with CF; i.e. the morphologically plastic *Flectobacillus* cluster (Hahn*, et al.*, 1999, Corno & Jürgens, 2006, Šimek*, et al.*, 2007) and permanently filamentous bacteria related to *Saprospiraceae* (Pernthaler, 2005, Schauer & Hahn, 2005, Schauer*, et al.*, 2006). The LD2 group ('*Candidatus* Aquirestis calciphila' (Hahn & Schauer, 2007)) of the *Saprospiraceae* are commonly found in Lake Zurich during the spring bloom, typically when grazing pressure is enhanced (*Articles I&II*). Experimental evidence suggests that these bacteria profit from the

presence of flagellated grazers, and their abundance is reduced when daphnids are present (Pernthaler, *et al.*, 2004, Schauer, *et al.*, 2006).

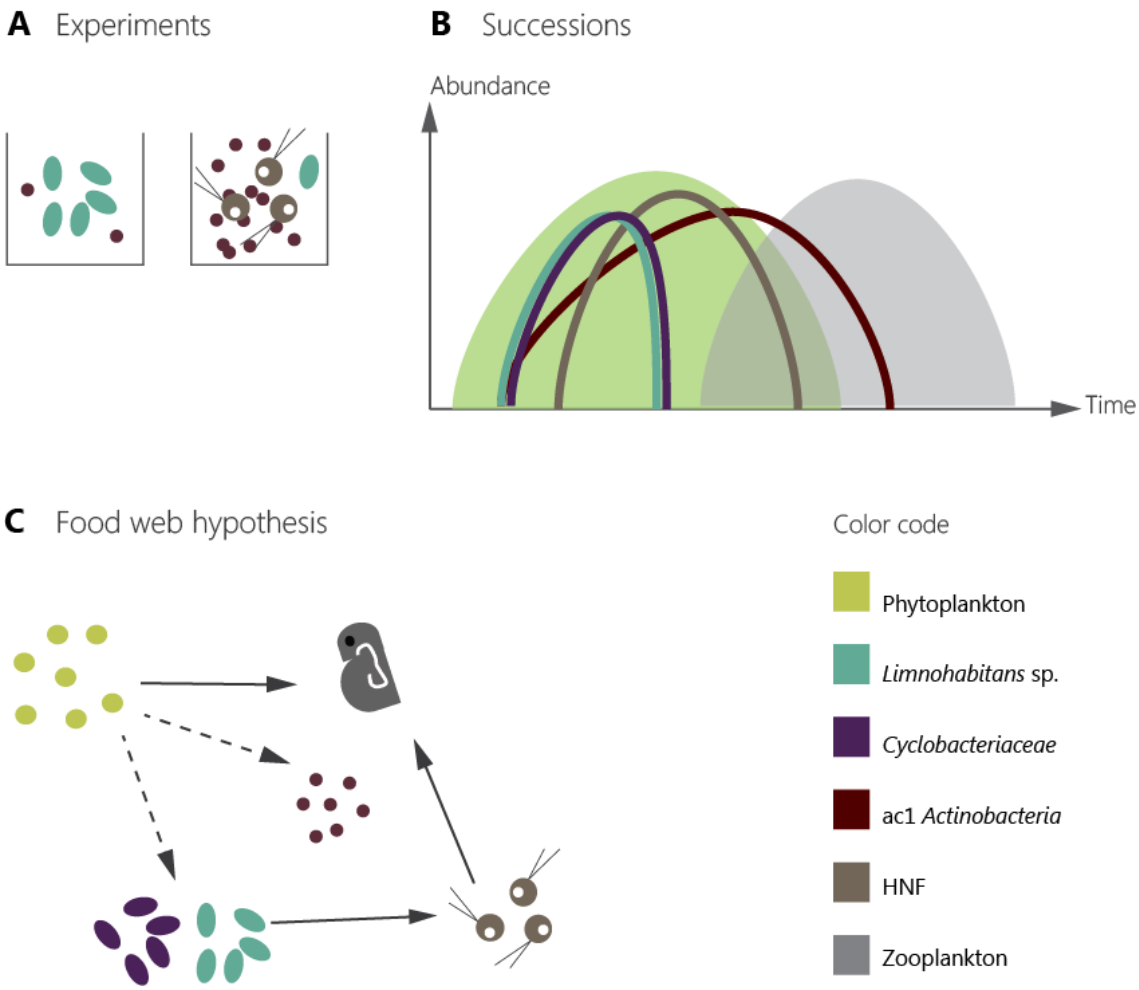


Fig 2 Schematic depiction of how experimental data, and observation of successions help to create food web hypotheses. (A) Experimental enrichments of Lhb or ac1 bacteria, with and without flagellates, respectively (based on Šimek et al. (2005), Pernthaler et al. (2001)). (B) Seasonal successions of Phytoplankton Lhb, *Cyclobacteriaceae*, HNF, ac1 and zooplankton (weakly based on *Article I*), (C) Food web interaction scheme for the species depicted in (B), where phytoplankton served as DOC source for bacteria (dashed line), HNF feed on Lhb and *Cyclobacteriaceae*, and *Daphnia* feed on phytoplankton and HNF.

Observations of the development of a bacterial population in nature may indicate whether a bacterial group is more strongly top-down or bottom-up controlled: short-lived, fast-growing populations are likely consumed by grazers whereas populations appearing at a later stage, and that increase concomitantly with bacterivorous flagellates, are rather specialised in avoiding this top-down control. Thus, prior experimentally collected knowledge

allows formulating a hypothesis about the adaptation of bacterial populations and their placement in the food web (Fig 2). By means of observation of their abundance we may weigh the importance of the observed patterns, since in a manipulation experiment usually only one or two factors of a habitat are targeted (such as HNF grazing and phosphorous availability e.g. Salcher*, et al.*, 2007) whereas the rest of the system is assumed to be constant. These changes may favour a certain population, which does not necessarily imply that the manipulated factors are the most defining ones in nature where many aspects interact (Strom, 2008).

Compared to a steady-state system, the above mentioned pronounced temporal fluctuation of abundances during phytoplankton blooms might be, as argued by Carpenter, a '*source of frustration*' for ecologists (Carpenter, 1988). On the other hand, the rapid turnovers allow to observe the dynamics of the systems, i.e. the dynamics of abundance of specific organismic groups in relation to other fluctuating factors, which may help to explain the role of certain species in the food web (Carpenter, 1988, Crowder*, et al.*, 1988). However, a high resolution of sampling points is required to elucidate the maximum abundances of species in such short lived patterns (Chesson & Huntly, 1997).

For example, in the sampling campaign conducted during the spring bloom 2009 in Lake Zurich (*Article I*), we observed various populations of bacteria amongst them Lhb and ac1 (*Article I*, Fig 2). Comparing their abundance at the initiation of the bloom, Lhb abundances rose very fast, but subsequently decreased at the time point of maximum HNF grazers. This is in line with the experimental hypothesis that Lhb profit from algal derived exudates but seem to be preferentially consumed by flagellates (Šimek*, et al.*, 2005, Šimek*, et al.*, 2011). Ac1 abundances rose parallel to Lhb in the beginning of the bloom, however, they did not decrease with rising HNF abundances. On the contrary, the HNF maxima always corresponded to the ac1 maxima, which supports the previous observations of ac1 not being consumed by HNF (Pernthaler*, et al.*, 2001). In this study we also analysed bacterial taxa for which no prior experimental data existed, such as the genus *Cyclobacteriaceae* of CF, which were rather prominent in abundance. Comparing their abundance pattern to groups where previous data exist, the abundance pattern of *Cylclobacteriaceae* highly resembled the one of Lhb. Moreover, microscopic images suggest a similar size of the two bacterial taxa (Article I). This opportunistic growth behaviour may reflect a fast uptake of organic carbon, and the disappearance of the group with increasing abundances of HNF hints to top-down control of

theses bacteria. Thus we hypothesize that these bacteria also profit from algal exudates but are consumed by flagellates, which allows us to place them into a food web scheme alongside other bacterial taxa (Fig 2). In other words, observation of successions may be translated into hypothesis of the ecological niche of i.e. *Cyclobacteriaceae*.

2 INTERPLAY OF BOTTOM-UP AND TOP-DOWN FACTORS

Disentangling bottom-up from top-down factors may be an appropriate first attempt to define the niche space of prokaryotic populations. In nature, however, these factors appear concomitantly and are also mechanistically linked. For example *Daphnia* spp. feed on algae and filamentous bacterial cells but also regenerate nutrients due to sloppy feeding, which stimulates microbial growth (Riemann*, et al.*, 1986, Peduzzi & Herndl, 1992). Similarly, HNF are the main consumers of heterotrophic bacteria (Porter*, et al.*, 1985, Posch*, et al.*, 1999). At the same time, HNF egest part of the pre-digested prey back to the water body which is again available as a substrate for bacteria (Goldman & Caron, 1985, Nagata & Kirchman, 1992). This comprises substrates such as the bacterial cell wall component peptidoglycan or membrane polymers (Nagata & Kirchman, 1992, Nagata & Kirchman, 2000). Thus HNF function as predator and simultaneously shape the DOC pool.

The evidence of HNF egestion published in the 1990's and early 2000's (see also (Nagata & Kirchman, 1997) and references therein) largely escaped the attention of studies of the last decade, when research focused much more on e.g. genotypic and phenotypic successions or aggregation as a response to grazing (e.g. Hahn*, et al.*, 2004, Corno*, et al.*, 2008, Blom*, et al.*, 2010). Inspired by a shift in N-acetyl-glucosamine (NAG) uptake observed during the spring phytoplankton bloom (*Article I*) we formulated a hypothesis for a possible explanation of the success of ac1 and LD2 (*Article II*). Microbial NAG incorporation during the spring bloom 2009 was first predominantly assigned to fast-growing CF and Beta; thereafter it shifted towards a dominance of ac1, in parallel to increasing HNF grazing pressure. Consequently we investigated whether ac1 and the protistan grazing-resistant filamentous LD2 bacteria might profit from NAG related to polymers that can be released through flagellate grazing, such as peptidoglycan (Nagata & Kirchman, 1992). *Article II* presents evidence obtained for a high uptake of NAG by these bacteria and a relationship of this uptake with the structural polymers peptidoglycan and chitin. Our data suggest that ac1 and LD2 profit from both, the

top-down and the bottom-up influence of HNF: firstly by the removal of competitors, and secondly by the supply of substrate.

We may speculate about the coexistence of ac1 and LD2. A recent study proposed that the coexistence of freshwater bacteria may also be mediated via partitioning of substrate niches (Salcher*, et al.*, 2013). While compelling for many bacterioplankton taxa, the substrate uptake patterns of LD2 and ac1 do not seem to greatly differ: In Salcher et al. (2013) these two bacterial groups showed nearly identical preferences for 14 low molecular weight organic carbon substrates (with the exception of fructose which was only taken up by filamentous bacteria). Furthermore, their shared resistance to HNF grazing and the successful enrichment of both groups on the same organic carbon compounds (*Article II*) seem to suggest a considerable niche-overlap. Nevertheless, substantial co-occurring populations of LD2 and ac1 are commonly found in temperate lakes during late spring.

However, a closer look at their respective vernal population development in Lake Zurich reveals that ac1 usually formed major populations shortly before LD2 were detectable (*Articles I&II*), suggesting that these *Actinobacteria* may be faster in substrate acquisition than the filaments. In our enrichment experiments, LD2 usually persisted longer in the system than ac1 (*Article II*). Considering a "*killing the winner*" scenario (Thingstad & Lignell, 1997, Winter*, et al.*, 2010), it is conceivable that ac1, which constituted more than 20% of the bacterial community, and had more than one order of magnitude higher cell densities than LD2, were more affected by viral infections. Very small bacteria commonly dominate the aquatic community (Posch*, et al.*, 2009). Next to ac1, LD12 Alpha-*Proteobacteria* are the most abundant group of epilimnetic freshwater bacteria, and the pelagial of the ocean is dominated by their sister clade '*Candidatus* Pelagibacter ubique' *(Morris, et al., 2002, Salcher, et al., 2011)*. It has been shown that bacteria related to '*Cand*. P. ubique' are highly affected by viral lysis (Zhao*, et al.*, 2013). If the population of ac1 were also constrained in growth due to viral lysis, this would again release peptidoglycan that could, in turn, provide substrate for LD2 (Middelboe & Jørgensen, 2006). Thus it is conceivable that viral lysis might transiently promote the coexistence of the two grazing-resistant bacterial groups (Riemann & Middelboe, 2002). Similar thoughts have been suggested by Šimek and co-workers (Šimek*, et al.*, 2007) to explain the success of another genus of filamentous pelagic bacteria (*Flectobacillus sp*.) that also seemed to particularly profit from high viral abundances.

Despite similar mechanism have been illustrated in a model system (Middelboe*, et al.*, 2003), this explanation for the coexistence of ac1 and LD2 is highly speculative. It solely illustrates a possible scenario of mortality influencing bacterial populations from top-down as much as from bottom-up. It is important to keep in mind that in microbial systems mortality and food supply are very tightly linked, particularly in the case of viral lysis (Töpper*, et al.*, 2013).

3 THE ROUTE OF A SUBSTRATE THROUGH THE MICROBIAL LOOP

The concept '*microbial loop*' was established for marine bacterioplankton in the early 1980's (Azam*, et al.*, 1983) and readily translates to freshwater ecosystems (Stockner & Porter, 1988). In essence it states that OC produced by exudation and lysis of phytoplankton is not lost in the dissolved pool but taken up by bacterioplankton which are consumed by protists such as flagellates or ciliates. These organisms are in turn eaten by zooplankton species and thereby the OC is channelled back to the classical food chain, thus to higher organisms.

NAG is incorporated by various microbial populations (*Articles I&II*). Since these groups do not share the same causes of mortality it may be speculated that the route of the substrate through the food web is not the same at all times. Combining the observations of high NAG uptake by grazing-resistant bacteria (*Article II*) and the shift in NAG uptake throughout the course of the spring bloom determined in *Article I*, an interesting pattern of NAG transfer through the food web arises: NAG may be preliminarily incorporated by fast-growing Beta and CF, which are fed on by HNF (Fig 3A). Thus, in the first part of the spring phytoplankton bloom NAG is transferred from fast-growing bacteria, e.g. CF and Beta, to flagellates which are grazed upon by ciliates and zooplankton. This corresponds to the above described pathway of the microbial loop (Azam*, et al.*, 1983).

Increased HNF abundances favour grazing-resistant bacteria, and flagellate egestion releases part of the incorporated NAG, which is taken up by ac1 and LD2. It is assumed that LD2 are mainly eaten by *Daphnia* spp. since their larger morphology allows being captured by the filter feeder (Pernthaler*, et al.*, 2004, Schauer*, et al.*, 2006). In this case the substrate re-enters the classical food chain directly at the trophic level of zooplankton, thereby circumventing the trophic level of protists (Fig 3B). In our third study we labelled lake bacterial assemblages with NAG and Leucine and let *Daphnia spp.* from Lake Zurich feed on

them (*Article III*). We found that a higher percentage of NAG labelled bacteria was incorporated by daphnids compared to leucine, which may be due to the substrate uptake by filamentous bacteria.

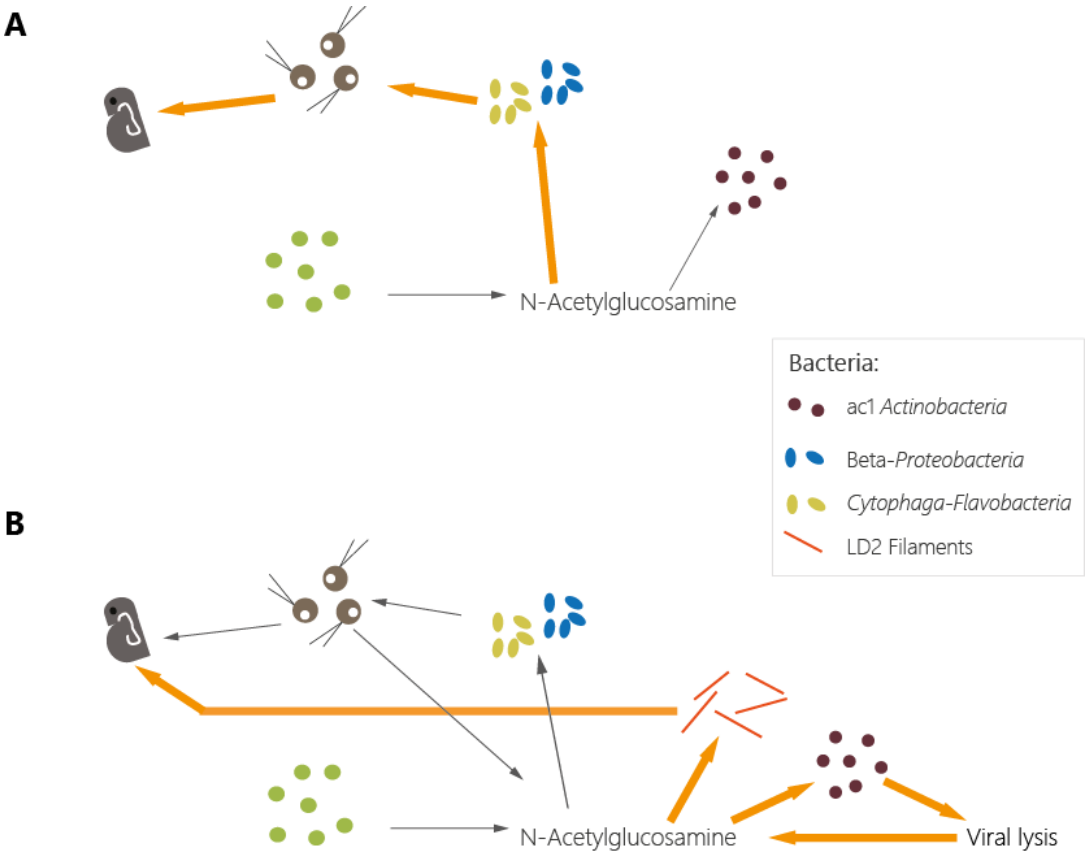


Fig 3 The transfer of N-acetyl-glucosamine (NAG) through the food web (based on *Articles I & II*). (A) NAG derives from phytoplankton exudation and is primarily incorporated by CF and Beta, which are eaten by HNF that are taken up by daphnids. (B) NAG derives from Phytoplankton and HNF egestion. It is incorporated by filamentous LD2 and small ac1. LD2 are grazed on by daphnids, whereas ac1 are lysed by viruses, thus the NAG returns to the pool of available substrate for heterotrophy.

As outlined above it is assumed that mortality of ac1 is mainly due to viral lysis (Weinbauer, *et al.*, 2007). Thus the NAG incorporated by ac1 is to a great extend transferred back to the pool of dissolved or colloidal organic carbon after lysis of the cells (Fig 3B). An important part of the microbial loop concept is that DOC is transferred to higher trophic levels though the uptake of bacteria, which is not the case for ac1. The role of ac1 is rather related to the viral shunt, where nutrients and OC are regenerated by viral lysis (Weinbauer, 2004). However, it has to be considered that every time carbon is sequestered into biomass part of it escapes the system through respiration (Pollard & Ducklow, 2011). A similar

scenario may apply for the above mentioned marine bacteria related to '*Candidatus* Pelagibacter ubique', which seem to be highly affected by viruses (Zhao*, et al.*, 2013). Therefore, the activity of ultramicrobacteria in the end may be a loss of energy for the ecosystem, and thus constitutes rather a sink than a link (Sherr*, et al.*, 1987)

The microbial loop has its name because it is attached to the classical food chain (Porter, *et al.*, 1988). However, considering biomass and energy turnover aspects of microbes compared to higher organisms, the classical food chain might be considered an appendage to the microbial trophic interactions (Pomeroy*, et al.*, 2007). Therein, however, the classical food chain seems to be only connected with some of the bacterial phylotypes and even dominant bacterial taxa, such as the ac1 or '*Cand*. P. ubique', may not directly form part of the microbial loop, since their biomass is short-circuited through viral lysis (Pollard & Ducklow, 2011).

4 INCLUDING EPIBIONTS INTO FOOD WEBS

Food webs are often depicted as networks, which are defined by nodes and their connections, where the nodes correspond to organismic groups, e.g. species, and the connections to the various interactions between them[3]. The structure of a food web determines the flow of matter, such as organic compounds, and its stability. For the understanding of the functioning of a food web species identity may be one important factor, the interaction, however, usually determines fluxes within the ecosystem. Thus detailed knowledge of interactions determines our possibility to forecast the impact of changes on the community.

Traditionally food web studies centre on trophic interaction, such as predation (Pimm, 1982, May, 1983, Petchey & Dunne, 2012, Thompson*, et al.*, 2012). This neglects many forms of species associations such as parasitism, and interactions which in bulk may be termed *non-trophic* (Kéfi*, et al.*, 2012, Sukhdeo, 2012), that in pelagic aquatic systems include, but are not limited to: symbiosis, co-aggregation, and epibiosis (e.g. Threlkeld & Willey, 1993, Simon*, et al.*, 2002, Corno*, et al.*, 2012).

Our third study explored the hypothesis that metabolically active bacteria attached to the surfaces of aquatic zooplankton might form a short-cut for DOC transfer in freshwater food

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[3] Stuart Pimm terms such food web depictions '*caricature of nature*', he however concludes that; '*Like any caricature though their representation of nature is distorted, there is enough truth to permit a study of some of the features they represent.*' (Pimm, Food Webs, 1982)

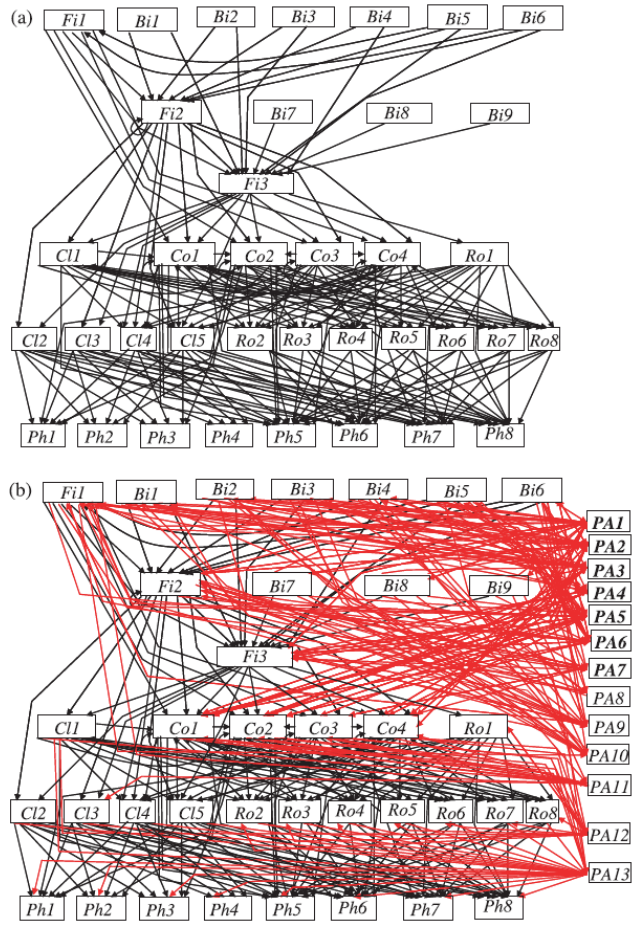

Fig 4 Representation of the trophic interactions in an artic lake food web of Birds (Bi), Fish (Fi), Copepods (Co), Rotifers (Ro), Cladocerans (Cl) and Phytoplankton (Ph) (a) without (black lines) and (b) including parasites (PA, red lines). Copied from Fig 1 Amudsen et al. (2009).

webs, since their biomass is directly transmitted to the next trophic level when their host is consumed (*Article III*). We analysed the uptake of model DOC compounds by the bacterial epibionts on *Daphnia* spp. both in cultures and in field samples. We found that a substantial amount of these compounds was incorporated by the epibiotic microbes. We furthermore identified an important epibiotic bacterial population on the filtration combs of the daphnids, where a constant supply of fresh water is guaranteed by the filtration activity of the animals. A major proportion of these bacteria were affiliated with Lhb and targeted by the probe R-Bt065 (see also Chapter 1). It has been repeatedly argued by ecologists that the discovery of such non-trophic interactions is a crucial next step for the understanding of natural freshwater systems (Tang, *et al.*, 2010, Kéfi, *et al.*, 2012, Mougi & Kondoh, 2012).

Most efforts to include non-traditional interactions into food web studies are taken in parasite ecology (Decaestecker, *et al.*, 2005, Lafferty, *et al.*, 2006). Amudsen et al. (2009) have constructed two food webs from a subarctic lake with and without parasites (Fig 4). Including parasites has led to a large increase of connectivity of the food web, a measure to evaluate how many of all possible connection between organisms are in fact realised. Thus parasites play an important role in shaping the food web. The network presented by the authors as well as other pioneer studies on parasites in food webs is, however, still based on predator prey relations, where parasites are handled as predators. Sukhdeo (2012) has recently argued that this may not be the appropriate approach to investigate these interactions. For example,

parasites cannot be considered a separate trophic level, since they gain their energy from the primary host, and thereby weaken the host. However, they also have a number of intermediate hosts which they do not necessarily influence negatively. Sukehdeo (2012) argues that a good starting point to include parasites more accurately in food webs may be to investigate their energetics and influence of parasites on the energy flow through the web, rather than just predator-prey interactions of different trophic levels. In fact, energetics is a classical topic of food web theory: Earliest studies have tried to elucidate the efficiency of energy transfer from one trophic level to the other, and came up with the *rule of 10*, which states, that only 10% of energy from a given trophic level is transferred to the next one (Lindeman, 1942). Interestingly, despite the simplification and the age of this theory it in fact still holds true for the majority of ecosystems, and it seems that even many parasitic nematodes may more or less show this efficiency when infecting a host (Sukhdeo, 2012).

The argument of using energetics may even weigh stronger when considering epibionts, since compared to parasites, the energy of epibionts may be completely uncoupled from the host. Using the example of *Daphnia* and two of its epibionts, I would like to illustrate the importance of including energetics in the case of epibiosis. Next to bacterial epibionts, such as Lhb, it has long been known that *Daphnia spp*. are often colonised by eukaryotic organisms such as flagellates or ciliates of the group Peritrichia (Threlkeld*, et al.*, 1993). Peritrichs are mostly sessile ciliate species on a contractile stalk but they can temporarily also live in planktonic form. Their main diet is comprised by bacteria which are transported into their mouth cavity by the movements of their cilia (Foissner, 1992). Thirty-nine species of Peritrichia are known to be epibionts of cladocerans (Chatterjee*, et al.*, 2013). On *Daphnia spp*. most studies have been carried out with members of the genus *Vorticella*, which were found on almost 50% of *D. magna* when natural populations were analysed (Decaestecker*, et al.*, 2005).

In the classical food web matrix every organism that feeds on another one is given a value of 1, whereas not feeding is valued 0. Thus a classical food web matrix containing a fish, daphnids, peritrichs, Lhb bacteria, algae and DOC as a food source for bacteria would be composed as in Figure 5A. A food web is then constructed where an interaction is drawn if there is predation between the two organisms (Fig 5B). Thus fish feed on daphnids that feed on protists and algae, and the latter provide DOC for bacteria that are incorporated by protists (Fig 5B). If, however, epibionts are considered the scheme changes: It is conceivable

that when a fish feeds on a daphnid it automatically also ingests the Peritrichia and Lhb bacteria attached to the organism (Fig 5C). Thus the fish does not feed on a single organism but on an aggregate of species, traditionally considered separated trophic levels. Nevertheless, all of these species may contribute to the maintenance of the fish's biomass. Our results show that the direct transfer of DOC to zooplankton via their epibiotic bacteria may be orders of magnitude higher than via the classical microbial food web, since no energy is lost during the passage through intermediate trophic levels (Fig 5D). Thus, the transfer of DOC to fish is much more efficient via epibionts.

Secondly, next to hidden trophic interactions, attached organisms also influence the energetics of the host and vice versa. For example, a high number of large epibionts, such as Peritrichia, can have a negative effect on the daphnids, for example faster sinking rates and higher energy demand for swimming (Henebry & Ridgeway, 1979). On the other hand, attachment seems to be beneficial to the stalked ciliates, since they have higher feeding rates when on zooplankton (Bickel*, et al.*, 2012). Bacteria may benefit from zooplankton as a refuge from threads such as grazing and abiotic stressors (Tang*, et al.*, 2010, Tang*, et al.*, 2011), or use zooplankton as means of transport from deeper to upper lake layers (Grossart*, et al.*, 2010). These bacteria may save energy in motility, however they also feed on two DOC pools that, due to lake stratification, are separated throughout a long time of the year, and belong to different basic food webs. Moreover attached bacteria may also directly profit from sloppy feeding of the crustacean, due to their vicinity to the mouth (Carman & Dobbs, 1997).

These ideas are not limited to *Daphnia spp*. epibionts, they also expand to many other zooplankton species in freshwaters (Grossart*, et al.*, 2009) as well as marine environments (Huq*, et al.*, 1983, Carman & Dobbs, 1997). Furthermore bacteria also attach to other organismic groups such as phytoplankton (Eigemann*, et al.*, 2013), and there are also indirect interactions such as free-living bacteria that promote the growth of algae (Grossart, 1999, Grossart*, et al.*, 2005).

As mentioned above, energetics was taken as a very early factor for the analysis of food webs. In his classic book 'food webs' Pimm (1982) suggested that energetics is not the right measure to evaluate these systems, since environmental data did not support the assumption. His main argument was that if energetics was the most important factor shaping a food web, more trophic levels should be observed in more productive regions (e.g.

eutrophic vs. oligotrophic lakes). According to the author, food chains rarely increased in length with

increasing productivity in natural systems. However it has to be considered that many interactions were not known or not considered at the time, and as outlined above, many trophic levels may be aggregated and evaluated as a single entity. Thus with the awareness of these interactions the evaluations of the system may markedly differ. These findings have the potential to change our perception of carbon transfer within freshwater ecosystems.

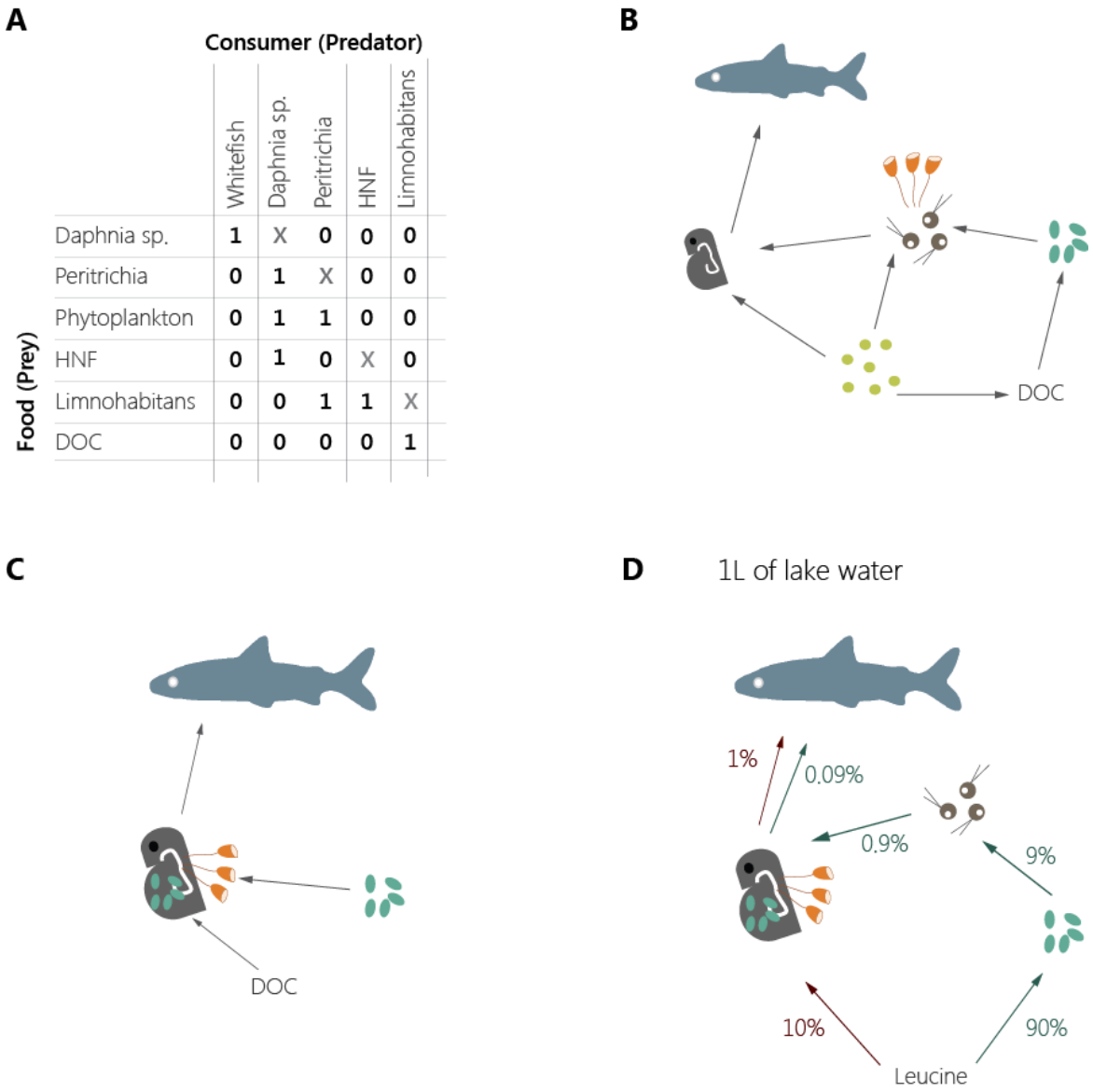

Fig 5 (A) Classical predator-prey matrix for whitefish, *Daphnia*, Peritrichia, Phytoplankton, Lhb and DOC and (B) the resulting food web. (C) Food web connection of *Daphnia*, epibionts and fish. (D) Example of the percentage of leucine transferred in 1L of lake water to fish via epibionts (dark red arrows) and the classical food web (blue arrows), when assuming a 10% efficiency of every trophic level (see Article III).

5 How the choice of method affects the outcome of a study

The successions described in *Article I* were determined using fluorescence *in situ* hybridisation (CARD-FISH). This method allows staining cells of a certain phylogenetic group using oligonucleotide probes with a signature sequence of a specific phylogenetic cluster. In this last section of the thesis I wish to show that the outcome of the study of spring bloom succession may have been cosiderably different if we had chosen another method for the evaluation of the bacterial community.

When using FISH, samples are assessed with a microscope, thus labelled cells are counted directly. This allows observing morphologies and directly determining counts of a specific phylogenetic group. The resolution of the phylogenetic analysis, however, is directly dependent on the possibility of distinguishing a group via their 16S rRNA sequence. If the targeted sequence region is too variable or too similar to other organismic groups, no probe can be constructed and the cluster will remain unstudied. In many cases a probe for a broader phylogenetic cluster may be designed that includes multiple phylogenetic lines. This can be problematic since taxa within the broader phylogenetic lineages may behave differently (Salcher, *et al.*, 2008, Salcher, *et al.*, 2013). Thus information about successions or physiology may be lost.

Alternatively sequencing methods may be used to evaluate the diversity of bacteria. In this case DNA is extracted from the bulk community and 16SrDNA sequences are PCR amplified and elucidated using one of various next-generation sequencing approaches (e.g. Illumina, 454-sequencing) (Eiler, *et al.*, 2011, Shokralla, *et al.*, 2012). For the spring bloom analysis 454 sequencing was conducted in parallel to FISH by cooperation partners (unpublished data kindly provided by Stefan Bertilsson).

Figure 6 depicts the relative abundance of two bacterial taxa firstly analysed with 454 pyrosequencing and secondly analysed by FISH. The upper panel shows the abundance of LD12 of Alpha-*Proteobacteria* assessed with both methods. LD12 were the most abundant bacterial phyla in the 454-analysis, where they comprised up to 20% of all prokaryotes, whereas in FISH they only represented 4%. Moreover, the temporal dynamics differ profoundly between the approaches, e.g. on April 24th LD12 had a maximum in the 454 analysis and a minimum in FISH. FISH analysis generally suggests that these bacteria rather have maxima in summer (Salcher, *et al.*, 2011). The contracdictory temporal patterns are even more visible in the analysis of ac1 (Fig 6, lower panel). These bacteria constituted a mayor

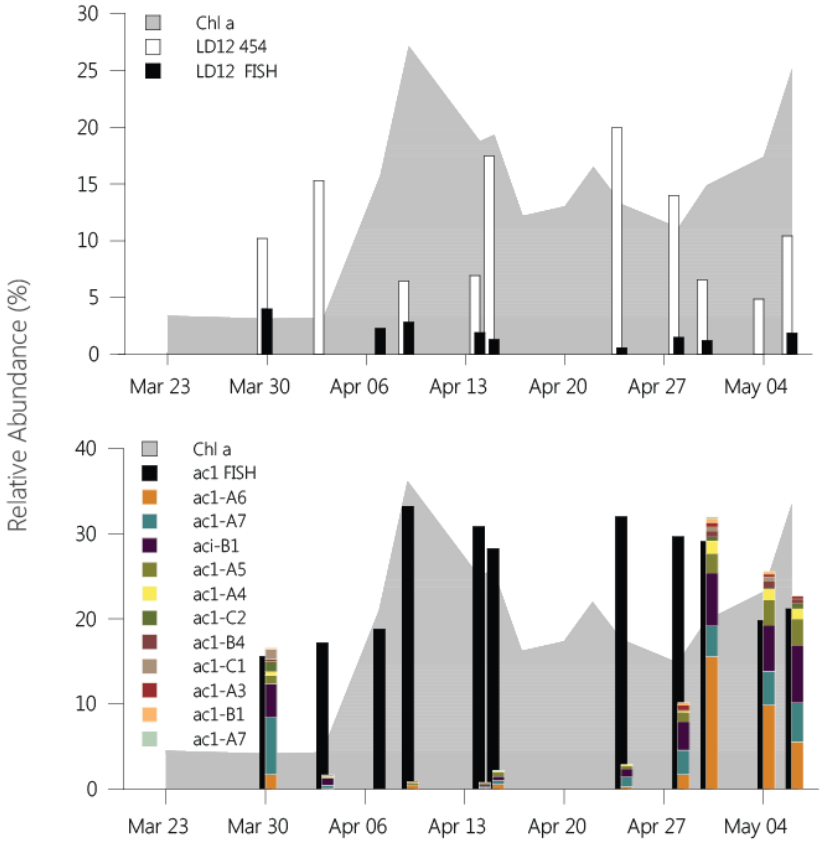

Fig 6 Comparison of abundance and diversity data determined with 454 pyrosequencing and FISH. Upper panel: Abundance of LD12, Lower panel: abundance of ac1 *Actinobacteria*. FISH: ac1: Article I, LD12: unpublished data, 454 data: S. Bertilsson unpublished. Sampling dates with less than 600 sequencing reads were omitted from the analysis

fraction of the bacteria analyzed by FISH during times of elevated chlorophyll *a*. In contrast they were of minor importance within the 454 reads between April 4th and 20th. 454 Pyrosequencing is usually not claimed to be a quantitative measure of analysis, due to the bias induced during PCR amplification, DNA extraction, and different 16S rRNA gene copy numbers (Farrelly, *et al.*, 1995, Wintzingerode, *et al.*, 1997, Frostegard, *et al.*, 1999). However, many authors do correlate relative 454 abundances with other parameters, to explain factors influencing the dynamics of the populations (Eiler, *et al.*, 2011). It is obvious that 454 and FISH data would deliver very different correlation patterns because of their profoundly different dynamics.

On the other hand it is clear that the group analysed as ac1 in FISH is actually comprised by a variety of separate taxa (Fig 6, lower panel). Thus certain patterns assigned to ac1 as a total group may in fact only be assigned to a part of the population, or are only visible due to a community shift within the ac1. Thus, every method is accompanied by specific draw-backs that need to be taken into account by the researcher.

In science there is still the claim of objectivity of the data. Examples such as the above-mentioned, however, clearly illustrate that we take decisions as scientist that influence the outcome of our studies, such as the choice of methods. This discussion has a long tradition in humanities (e.g. Ebeling & Schmitz, 2006), whereas the dispute hardly takes place among natural scientists. To my opinion, awareness of this fact is not a weakness of the scientific mind, since questioning everything has often pathed the way to new discoveries.

6 References

Allgaier M & Grossart HP (2006) Diversity and seasonal dynamics of Actinobacteria populations in four lakes in northeastern Germany. *Applied and Environmental Microbiology* 72: 3489-3497.

Allgaier M & Grossart HP (2006) Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany. *Aquatic Microbial Ecology* 45: 115-128.

Amundsen P-A, Lafferty KD, Knudsen R, Primicerio R, Klemetsen A & Kuris AM (2009) Food web topology and parasites in the pelagic zone of a subarctic lake. *Journal of Animal Ecology* 78: 563-572.

Azam F, Fenchel T, Field JG, Gray JS, Meyerreil LA & Thingstad F (1983) The ecological role of water-column microbes in the sea. *Marine Ecology-Progress Series* 10: 257-263.

Bickel SL, Tang KW & Grossart H-P (2012) Ciliate epibionts associated with crustacean zooplankton in German lakes: Distribution, motility and bacterivory. *Frontiers in Microbiology* 3.

Blom JF, Zimmermann YS, Ammann T & Pernthaler J (2010) Scent of Danger: Floc Formation by a Freshwater Bacterium Is Induced by Supernatants from a Predator-Prey Coculture. *Applied and Environmental Microbiology* 76: 6156-6163.

Bossard P, Gammeter S, Lehmann C*, et al.* (2001) Limnological description of the Lakes Zurich, Lucerne, and Cadagno. *Aquatic Sciences* 63: 225-249.

Boucher D, Jardillier L & Debroas D (2006) Succession of bacterial community composition over two consecutive years in two aquatic systems: a natural lake and a lake-reservoir. *Fems Microbiology Ecology* 55: 79-97.

Carman KR & Dobbs FC (1997) Epibiotic microorganisms on copepods and other marine crustaceans. *Microscopy Research and Technique* 37: 116-135.

Carpenter SR (1988) Transmission of Variance through Lake Food Webs. *Complex Interactions in Lake Communities,*(Carpenter SR), 119-136. Springer New York.

Chatterjee T, Kotov A & Fernández-Leborans G (2013) A checklist of epibiotic ciliates (Peritrichia and Suctoria) on the cladoceran crustaceans. *Biologia* 68: 439-447.

Chesson P & Huntly N (1997) The Roles of Harsh and Fluctuating Conditions in the Dynamics of Ecological Communities. *The American Naturalist* 150: 519-553.

Corno G & Jürgens K (2006) Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. *Applied and Environmental Microbiology* 72: 78-86.

Corno G & Jürgens K (2008) Structural and functional patterns of bacterial communities in response to protist predation along an experimental productivity gradient. *Environmental Microbiology* 10: 2857-2871.

Corno G, Villiger J & Pernthaler J (2012) Coaggregation in a microbial predator–prey system affects competition and trophic transfer efficiency. *Ecology* 94: 870-881.

Corno G, Caravati E, Callieri C & Bertoni R (2008) Effects of predation pressure on bacterial abundance, diversity, and size-structure distribution in an oligotrophic system. *Journal of Limnology* 67: 107-119.

Crowder LB, Drenner RW, Kerfoot WC*, et al.* (1988) Food Web Interactions in Lakes. *Complex interactions in Lake Communities,*(Carpenter SR), 141-160. Springer New York.

Decaestecker E, Declerck S, Meester L & Ebert D (2005) Ecological implications of parasites in natural Daphnia populations. *Oecologia* 144: 382-390.

Ebeling S & Schmitz S (2006) Geschlechterforschung und Naturwissenschaften. VS Verlag.

Eigemann F, Hilt S, Salka I & Grossart HP (2013) Bacterial community composition associated with freshwater algae: species specificity vs. dependency on environmental conditions and source community. *Fems Microbiology Ecology* 83: 650-663.

Eiler A, Heinrich F & Bertilsson S (2011) Coherent dynamics and association networks among lake bacterioplankton taxa. *Isme Journal*.

Farrelly V, Rainey FA & Stackebrandt E (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S ribosomal-RNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* 61: 2798-2801.

Felip M, Pace ML & Cole JJ (1996) Regulation of planktonic bacterial growth rates: The effects of temperature and resources. *Microbial Ecology* 31: 15-28.

Foissner W (1992) *Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems: Peritrichia, Heterotrichida, Odontostomatida*. Bayerisches Landesamt für Wasserwirtschaft.

Frostegard A, Courtois S, Ramisse V*, et al.* (1999) Quantification of bias related to the extraction of DNA directly from soils. *Applied and Environmental Microbiology* 65: 5409-5420.

Glöckner FO, Zaichikov E, Belkova N, Denissova L, Pernthaler J, Pernthaler A & Amann R (2000) Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. *Applied and Environmental Microbiology* 66: 5053-5065.

Goldman JC & Caron DA (1985) Experimental studies on an omnivorous microflagellate - implications for grazing and nutrient regeneration in the marine microbial food-chain. *Deep-Sea Research Part a-Oceanographic Research Papers* 32: 899-915.

Grossart HP (1999) Interactions between marine bacteria and axenic diatoms (Cylindrotheca fusiformis, Nitzschia laevis, and Thalassiosira weissflogii) incubated under various conditions in the lab. *Aquatic Microbial Ecology* 19: 1-11.

GROSSART HP, DZIALLAS C & TANG KW (2009) BACTERIAL DIVERSITY ASSOCIATED WITH FRESHWATER ZOOPLANKTON. *ENVIRONMENTAL MICROBIOLOGY REPORTS* 1: 50-55.

GROSSART HP, KIORBOE T, TANG K & PLOUG H (2003) BACTERIAL COLONIZATION OF PARTICLES: GROWTH AND INTERACTIONS. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 69: 3500-3509.

GROSSART HP, TANG KW, KIØRBOE T & PLOUG H (2007) COMPARISON OF CELL-SPECIFIC ACTIVITY BETWEEN FREE-LIVING AND ATTACHED BACTERIA USING ISOLATES AND NATURAL ASSEMBLAGES. *FEMS MICROBIOLOGY LETTERS* 266: 194-200.

GROSSART HP, DZIALLAS C, LEUNERT F & TANG KW (2010) BACTERIA DISPERSAL BY HITCHHIKING ON ZOOPLANKTON. *PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA* 107: 11959-11964.

GROSSART HP, LEVOLD F, ALLGAIER M, SIMON M & BRINKHOFF T (2005) MARINE DIATOM SPECIES HARBOUR DISTINCT BACTERIAL COMMUNITIES. *ENVIRONMENTAL MICROBIOLOGY* 7: 860-873.

HAHN MW & HÖFLE MG (2001) GRAZING OF PROTOZOA AND ITS EFFECT ON POPULATIONS OF AQUATIC BACTERIA. *FEMS MICROBIOLOGY ECOLOGY* 35: 113-121.

HAHN MW & SCHAUER M (2007) 'CANDIDATUS AQUIRESTIS CALCIPHILA' AND 'CANDIDATUS HALISCOMENOBACTER CALCIFUGIENS, FILAMENTOUS, PLANKTONIC BACTERIA INHABITING NATURAL LAKES. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY* 57: 936-940.

HAHN MW, MOORE ERB & HÖFLE MG (1999) BACTERIAL FILAMENT FORMATION, A DEFENSE MECHANISM AGAINST FLAGELLATE GRAZING, IS GROWTH RATE CONTROLLED IN BACTERIA OF DIFFERENT PHYLA. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 65: 25-35.

HAHN MW, LUNSDORF H & JANKE L (2004) EXOPOLYMER PRODUCTION AND MICROCOLONY FORMATION BY PLANKTONIC FRESHWATER BACTERIA: DEFENCE AGAINST PROTISTAN GRAZING. *AQUATIC MICROBIAL ECOLOGY* 35: 297-308.

HENEBRY MS & RIDGEWAY BT (1979) EPIZOIC CILIATED PROTOZOA OF PLANKTONIC COPEPODS AND CLADOCERANS AND THEIR POSSIBLE USE AS INDICATORS OF ORGANIC WATER POLLUTION. *TRANSACTIONS OF THE AMERICAN MICROSCOPICAL SOCIETY* 98: 495-508.

HUQ A, SMALL EB, WEST PA, HUQ MI, RAHMAN R & COLWELL RR (1983) ECOLOGICAL RELATIONSHIPS BETWEEN VIBRIO CHOLERAE AND PLANKTONIC CRUSTACEAN COPEPODS. *APPL ENVIRON MICROBIOL* 45: 275-283.

JEZBERA J, HORŇÁK K & ŠIMEK K (2005) FOOD SELECTION BY BACTERIVOROUS PROTISTS: INSIGHT FROM THE ANALYSIS OF THE FOOD VACUOLE CONTENT BY MEANS OF FLUORESCENCE IN SITU HYBRIDIZATION. *FEMS MICROBIOLOGY ECOLOGY* 52: 351-363.

JÜRGENS K, PERNTHALER J, SCHALLA S & AMANN R (1999) MORPHOLOGICAL AND COMPOSITIONAL CHANGES IN A PLANKTONIC BACTERIAL COMMUNITY IN RESPONSE TO ENHANCED PROTOZOAN GRAZING. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 65: 1241-1250.

KÉFI S, BERLOW EL, WIETERS EA, *ET AL.* (2012) MORE THAN A MEAL... INTEGRATING NON-FEEDING INTERACTIONS INTO FOOD WEBS. *ECOLOGY LETTERS* 15: 291-300.

KENT AD, YANNARELL AC, RUSAK JA, TRIPLETT EW & MCMAHON KD (2007) SYNCHRONY IN AQUATIC MICROBIAL COMMUNITY DYNAMICS. *ISME JOURNAL* 1: 38-47.

KIØRBOE T, GROSSART H-P, PLOUG H, TANG K & AUER B (2004) PARTICLE-ASSOCIATED FLAGELLATES: SWIMMING PATTERNS, COLONIZATION RATES, AND GRAZING ON ATTACHED BACTERIA. *AQUATIC MICROBIAL ECOLOGY* 35: 141-152.

LAFFERTY KD, DOBSON AP & KURIS AM (2006) PARASITES DOMINATE FOOD WEB LINKS. *PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA* 103: 11211-11216.

LANGENHEDER S & JÜRGENS K (2001) REGULATION OF BACTERIAL BIOMASS AND COMMUNITY STRUCTURE BY METAZOAN AND PROTOZOAN PREDATION. *LIMNOLOGY AND OCEANOGRAPHY* 46: 121-134.

LINDEMAN RL (1942) THE TROPHIC-DYNAMIC ASPECT OF ECOLOGY. *ECOLOGY* 23: 399-418.

MANZ W, AMANN R, LUDWIG W, VANCANNEYT M & SCHLEIFER KH (1996) APPLICATION OF A SUITE OF 16S RRNA-SPECIFIC OLIGONUCLEOTIDE PROBES DESIGNED TO INVESTIGATE BACTERIA OF THE PHYLUM CYTOPHAGA-FLAVOBACTER-BACTEROIDES IN THE NATURAL ENVIRONMENT. *MICROBIOLOGY-UK* 142: 1097-1106.

MAY RM (1983) ECOLOGY: THE STRUCTURE OF FOOD WEBS. *NATURE* 301: 566-568.

MIDDELBOE M & JØRGENSEN NOG (2006) VIRAL LYSIS OF BACTERIA: AN IMPORTANT SOURCE OF DISSOLVED AMINO ACIDS AND CELL WALL COMPOUNDS. *JOURNAL OF THE MARINE BIOLOGICAL ASSOCIATION OF THE UNITED KINGDOM* 86: 605-612.

MIDDELBOE M, RIEMANN L, STEWARD GF, HANSEN V & NYBROE O (2003) VIRUS-INDUCED TRANSFER OF ORGANIC CARBON BETWEEN MARINE BACTERIA IN A MODEL COMMUNITY. *AQUATIC MICROBIAL ECOLOGY* 33: 1-10.

MORRIS RM, RAPPE MS, CONNON SA, VERGIN KL, SIEBOLD WA, CARLSON CA & GIOVANNONI SJ (2002) SAR11 CLADE DOMINATES OCEAN SURFACE BACTERIOPLANKTON COMMUNITIES. *NATURE* 420: 806-810.

MOUGI A & KONDOH M (2012) DIVERSITY OF INTERACTION TYPES AND ECOLOGICAL COMMUNITY STABILITY. *SCIENCE* 337: 349-351.

NAGATA T & KIRCHMAN DL (1992) RELEASE OF MACROMOLECULAR ORGANIC-COMPLEXES BY HETEROTROPHIC MARINE FLAGELLATES. *MARINE ECOLOGY-PROGRESS SERIES* 83: 233-240.

NAGATA T & KIRCHMAN DL (1997) ROLES OF SUBMICRON PARTICLES AND COLLOIDS IN MICROBIAL FOOD WEBS AND BIOGEOCHEMICAL CYCLES WITHIN MARINE ENVIRONMENTS. *ADVANCES IN MICROBIAL ECOLOGY,* VOL. 15 (JONES JG), 81-103. PLENUM PRESS DIV PLENUM PUBLISHING CORP, NEW YORK.

NAGATA T & KIRCHMAN DL (2000) BACTERIAL MORTALITY: A PATHWAY FOR THE FORMATION OF REFRACTORY DOM? *MICROBIAL BIOSYSTEMS: NEW FRONTIERS - PROCEEDINGS OF THE 8TH INTERNATIONAL SYMPOSIUM ON MICROBIAL ECOLOGY,*(BELL CR, BRYLINSKY M & JOHNSON-GREEN P), 153-158. ATLANTIC CANADA SOCIETY FOR MICROBIAL ECOLOGY, HALIFAX, CANADA.

Peduzzi P & Herndl GJ (1992) Zooplankton activity fueling the microbial loop - differential growth-response of bacteria from oligotrophic and eutrophic waters. *Limnology and Oceanography* 37: 1087-1092.

Pernthaler J (2005) Predation on prokaryotes in the water column and its ecological implications. *Nature Reviews Microbiology* 3: 537-546.

Pernthaler J, Zöllner E, Warnecke F & Jürgens K (2004) Bloom of filamentous bacteria in a mesotrophic lake: Identity and potential controlling mechanism. *Applied and Environmental Microbiology* 70: 6272-6281.

Pernthaler J, Posch T, Šimek K*, et al.* (2001) Predator-specific enrichment of actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture. *Applied and Environmental Microbiology* 67: 2145-2155.

Petchey OL & Dunne JA (2012) Predator–Prey Relations and Food Webs. *Metabolic Ecology: A Scaling Approach* 86.

Pimm SL (1982) *Food webs*. Springer Netherlands.

Pollard PC & Ducklow H (2011) Ultrahigh bacterial production in a eutrophic subtropical Australian river: Does viral lysis short-circuit the microbial loop? *Limnology and Oceanography* 56: 1115-1129.

Pomeroy LR, Williams PJI, Azam F & Hobbie JE (2007) The Microbial Loop. *Oceanography* 20: 28-33.

Porter KG, Sherr EB, Sherr BF, Pace M & Sanders RW (1985) Protozoa in Planktonic Food Webs. *Journal of Eukaryotic Microbiology* 32: 409-415.

Porter KG, Paerl H, Hodson R*, et al.* (1988) Microbial Interactions In Lake Food Webs. *Complex interactions in Lake Communities,*(Carpenter SR), 209-228. Springer New York.

Posch T, Franzoi J, Prader M & Salcher MM (2009) New image analysis tool to study biomass and morphotypes of three major bacterioplankton groups in an alpine lake. *Aquatic Microbial Ecology* 54: 113-126.

Posch T, Simek K, Vrba J*, et al.* (1999) Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. *Aquatic Microbial Ecology* 18: 235-246.

Riemann B, Jørgensen NOG, Lampert W & Fuhrman JA (1986) Zooplankton Induced Changes in Dissolved Free Amino Acids and in Production Rates of Freshwater Bacteria. *Microbial Ecology* 12: 247-258.

Riemann L & Middelboe M (2002) Viral lysis of marine bacterioplankton: Implications for organic matter cycling and bacterial clonal composition. *Ophelia* 56: 57-68.

Rusak JA, Jones SE, Kent AD, Shade AL & McMahon TM (2009) Spatial synchrony in microbial community dynamics: testing among-year and lake patterns. *International Association of Theoretical and Applied Limnology, Vol 30, Pt 6, Proceedings,* Vol. 30 (Jones J & Faaborg J), 936-940. E Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.

Salcher MM, Pernthaler J & Posch T (2010) Spatiotemporal distribution and activity patterns of bacteria from three phylogenetic groups in an oligomesotrophic lake. *Limnology and Oceanography* 55: 846-856.

Salcher MM, Pernthaler J & Posch T (2011) Seasonal bloom dynamics and ecophysiology of the freshwater sister clade of SAR11 bacteria 'that rule the waves' (LD12). *Isme Journal* doi:10.1038.

Salcher MM, Posch T & Pernthaler J (2013) In situ substrate preferences of abundant bacterioplankton populations in a prealpine freshwater lake. *Isme Journal* 7: 896-907.

Salcher MM, Pernthaler J, Frater N & Posch T (2011) Vertical and longitudinal distribution patterns of different bacterioplankton populations in a canyon-shaped, deep prealpine lake. *Limnology and Oceanography* 56: 2027-2039.

Salcher MM, Pernthaler J, Zeder M, Psenner R & Posch T (2008) Spatio-temporal niche separation of planktonic Betaproteobacteria in an oligo-mesotrophic lake. *Environmental Microbiology* 10: 2074-2086.

Salcher MM, Hofer J, Horňák K*, et al.* (2007) Modulation of microbial predator-prey dynamics by phosphorus availability: Growth patterns and survival strategies of bacterial phylogenetic clades. *Fems Microbiology Ecology* 60: 40-50.

Schauer M & Hahn MW (2005) Diversity and phylogenetic affiliations of morphologically conspicuous large filamentous bacteria occurring in the pelagic zones of a broad spectrum of freshwater habitats. *Applied and Environmental Microbiology* 71: 1931-1940.

Schauer M, Jiang J & Hahn MW (2006) Recurrent seasonal variations in abundance and composition of filamentous SOL cluster bacteria (Saprospiraceae, Bacteroidetes) in oligomesotrophic Lake Mondsee (Austria). *Applied and Environmental Microbiology* 72: 4704-4712.

Schweitzer B & Simon M (1995) Growth limitation of planktonic bacteria in a large mesotrophic lake. *Microbial Ecology* 30: 89-104.

Sherr EB, Sherr BF & Albright LJ (1987) Bacteria - Link or Sink. *Science* 235: 88-88.

Shokralla S, Spall JL, Gibson JF & Hajibabaei M (2012) Next- generation sequencing technologies for environmental DNA research. *Molecular Ecology* 21: 1794-1805.

Šimek K, Kasalický V, Zapomelova E & Horňák K (2011) Alga-Derived Substrates Select for Distinct Betaproteobacterial Lineages and Contribute to Niche Separation in Limnohabitans Strains. *Applied and Environmental Microbiology* 77: 7307-7315.

Šimek K, Weinbauer MG, Horňák K, Jezbera J, Nedoma J & Dolan JR (2007) Grazer and virus-induced mortality of bacterioplankton accelerates development of Flectobacillus populations in a freshwater community. *Environmental Microbiology* 9: 789-800.

Šimek K, Horňák K, Mašin M, Christaki U, Nedoma J, Weinbauer MG & Dolan JR (2003) Comparing the effects of resource enrichment and grazing on a bacterioplankton community of a meso-eutrophic reservoir. *Aquatic Microbial Ecology* 31: 123-135.

Šimek K, Horňák K, Jezbera J, Mašin M, Nedoma J, Gasol JM & Schauer M (2005) Influence of top-down and bottom-up manipulations on the R-BT065 subcluster of beta-proteobacteria, an abundant group in bacterioplankton of a freshwater reservoir. *Applied and Environmental Microbiology* 71: 2381-2390.

Simon M, Tilzer MM & Muller H (1998) Bacterioplankton dynamics in a large mesotrophic lake: I. Abundance, production and growth control. *Archiv Für Hydrobiologie* 143: 385-407.

Simon M, Grossart HP, Schweitzer B & Ploug H (2002) Microbial ecology of organic aggregates in aquatic ecosystems. *Aquatic Microbial Ecology* 28: 175-211.

Sommer U (1986) The periodicity of phytoplankton in Lake Constance (Bodensee) in comparison to other deep lakes of central Europe. *Hydrobiologia* 138: 1-7.

Stockner J & Porter K (1988) Microbial Food Webs in Freshwater Planktonic Ecosystems. *Complex Interactions in Lake Communities,*(Carpenter SR), 69-83. Springer New York.

Strom SL (2008) Microbial ecology of ocean biogeochemistry: A community perspective. *Science* 320: 1043-1045.

Sukhdeo MVK (2012) Where are the parasites in food webs? *Parasites & Vectors* 1-17.

Tang KW, Turk V & Grossart HP (2010) Linkage between crustacean zooplankton and aquatic bacteria. *Aquatic Microbial Ecology* 61: 261-277.

Tang KW, Dziallas C & Grossart HP (2011) Zooplankton and aggregates as refuge for aquatic bacteria: protection from UV, heat and ozone stresses used for water treatment. *Environmental Microbiology* 13: 378-390.

Tarao M, Jezbera J & Hahn MW (2009) Involvement of Cell Surface Structures in Size-Independent Grazing Resistance of Freshwater Actinobacteria. *Applied and Environmental Microbiology* 75: 4720-4726.

Thingstad TF & Lignell R (1997) Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. *Aquatic Microbial Ecology* 13: 19-27.

Thompson RM, Brose U, Dunne JA*, et al.* (2012) Food webs: reconciling the structure and function of biodiversity. *Trends in Ecology & Evolution* 27: 689-697.

Threlkeld ST & Willey RL (1993) Colonization, interaction, and organization of cladoceran epibiont communities. *Limnology and Oceanography* 38: 584-591.

Threlkeld ST, Chiavelli DA & Willey RL (1993) The organization of zooplankton epibiont communities. *Trends in Ecology & Evolution* 8: 317-321.

Töpper B, Thingstad TF & Sandaa R-A (2013) Effects of differences in organic supply on bacterial diversity subject to viral lysis. *Fems Microbiology Ecology* 83: 202-213.

Weinbauer MG (2004) Ecology of prokaryotic viruses. *Fems Microbiology Reviews* 28: 127-181.

Weinbauer MG & Höfle MG (1998) Distribution and life strategies of two bacterial populations in a eutrophic lake. *Applied and Environmental Microbiology* 64: 3776-3783.

Weinbauer MG, Horňák K, Jezbera J, Nedoma J, Dolan JR & Šimek K (2007) Synergistic and antagonistic effects of viral lysis and protistan grazing on bacterial biomass, production and diversity. *Environmental Microbiology* 9: 777-788.

Weisse T (1991) The annual cycle of heterotrophic freshwater nanoflagellates: role of bottom-up versus top-down control. *Journal of Plankton Research* 13: 167-185.

Winter C, Bouvier T, Weinbauer MG & Thingstad TF (2010) Trade-Offs between Competition and Defense Specialists among Unicellular Planktonic Organisms: the "Killing the Winner" Hypothesis Revisited. *Microbiology and Molecular Biology Reviews* 74: 42-57.

Wintzingerode FV, Goebel UB & Stackebrandt E (1997) Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *Fems Microbiology Reviews* 21: 213-229.

Zeder M, Peter S, Shabarova T & Pernthaler J (2009) A small population of planktonic Flavobacteria with disproportionally high growth during the spring phytoplankton bloom in a prealpine lake. *Environmental Microbiology* 11: 2676-2686.

Zhao YL, Temperton B, Thrash JC*, et al.* (2013) Abundant SAR11 viruses in the ocean. *Nature* 494: 357-360.

ARTICLE I

ECKERT EM, SALCHER MM, POSCH T, EUGSTER B & PERNTHALER J (2012) RAPID SUCCESSIONS AFFECT MICROBIAL N-ACETYL-GLUCOSAMINE UPTAKE PATTERNS DURING A LACUSTRINE SPRING PHYTOPLANKTON BLOOM. ENVIRONMENTAL MICROBIOLOGY 14: 794-806.

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Rapid successions affect microbial *N*-acetyl-glucosamine uptake patterns during a lacustrine spring phytoplankton bloom

Ester M. Eckert, Michaela M. Salcher, Thomas Posch, Bettina Eugster and Jakob Pernthaler*

Limnological Station, Institute of Plant Biology, University of Zurich, Seestrasse 187, 8802 Kilchberg, Switzerland.

Summary

The vernal successions of phytoplankton, heterotrophic nanoflagellates (HNF) and viruses in temperate lakes result in alternating dominance of top-down and bottom-up factors on the bacterial community. This may lead to asynchronous blooms of bacteria with different life strategies and affect the channelling of particular components of the dissolved organic matter (DOM) through microbial food webs. We followed the dynamics of several bacterial populations and of other components of the microbial food web throughout the spring phytoplankton bloom period in a pre-alpine lake, and we assessed bacterial uptake patterns of two constituents of the labile DOM pool (*N*-acetyl-glucosamine [NAG] and leucine). There was a clear genotypic shift within the bacterial assemblage, from fast growing *Cytophaga–Flavobacteria* (CF) affiliated with *Fluviicola* and from *Betaproteobacteria* (BET) of the *Limnohabitans* cluster to more grazing resistant AcI *Actinobacteria* (ACT) and to filamentous morphotypes. This was paralleled by successive blooms of viruses and HNF. We also noted the transient rise of other CF (related to *Cyclobacteriaceae* and *Sphingobacteriaceae*) that are not detected by fluorescence *in situ* hybridization with the general CF probe. Both, the average uptake rates of leucine and the fractions of leucine incorporating bacteria were approximately five to sixfold higher than of NAG. However, the composition of the NAG-active community was much more prone to genotypic successions, in particular of bacteria with different life strategies: While 'opportunistically' growing BET and CF dominated NAG uptake in the initial period ruled by bottom-up factors, ACT constituted the major fraction of NAG active cells during the subsequent phase of high predation pressure. This indicates that some ACT could profit from a substrate that might in parts have originated from the grazing of protists on their bacterial competitors.

Received 8 July, 2011; revised 4 October, 2011; accepted 13 October, 2011. *For correspondence. E-mail pernthaler@limnol.uzh.ch; Tel. (+41) 44 716 1210; Fax (+41) 44 716 1225.

Introduction

The success of bacteria affiliated with *Cytophaga–Flavobacteria* (CF) in the pelagic zone of aquatic habitats is believed to be closely related to the phytoplankton. CF have been found to rise in parallel with specific algal taxa (Fandino *et al.*, 2005; Eiler and Bertilsson, 2007; Zeder *et al.*, 2009) and they may be directly attached to phytoplankton cells (Abell and Bowman, 2005; Grossart *et al.*, 2005; Gómez-Pereira *et al.*, 2010). Abundances of CF in the Atlantic Ocean were correlated with chlorophyll *a* (Chl *a*) (Gómez-Pereira *et al.*, 2010), and these bacteria were most common in the surface waters of a lake during the spring algal bloom (Salcher *et al.*, 2010). Moreover, some freshwater CF are apparently stimulated by algal derived DOC and exhibit high growth rates even when low in abundance (Zeder *et al.*, 2009). However, the accurate assessment of the role of CF in freshwaters is currently hampered by a methodological problem: the abundances of these bacteria are often assessed by fluorescence *in situ* hybridization (FISH) with a group-specific oligonucleotide probe, CF-319a (Manz *et al.*, 1996). While the vast majority of marine CF are covered by this probe (Alonso *et al.*, 2007), this is not the case for important cosmopolitan freshwater lineages (Newton *et al.*, 2011), such as *Algoriphagus* spp. (Nedashkovskaya *et al.*, 2007) of *Cyclobacteriaceae* (Kumar *et al.*, 2010a).

Besides CF, other bacterial taxa are also of importance in lakes during the spring phytoplankton bloom period, when bacterial growth and respiration rates are typically high (Weisse *et al.*, 1990; Biddanda and Cotner, 2002). In fact, there is first evidence for characteristic successions of microbes with distinct growth strategies during this period: First, 'opportunistic' bacteria with large cell sizes and high growth potential closely follow the increase of algal biomass, but subsequently decline in abundance

due to their vulnerability to protistan grazing. Microbes affiliated with CF, but also with the genus *Limnohabitans* of *Betaproteobacteria* (BET) have been described to follow such patterns (Šimek *et al.*, 2001a; Eiler and Bertilsson, 2007; Jones *et al.*, 2009). Subsequently, the more slowly growing members of the ultramicrobacterial Ac1 lineage of *Actinobacteria* (ACT) are found to rise, during times of high abundances of bacterivorous nanoflagellates (Pernthaler *et al.*, 2001; Jezbera *et al.*, 2006; Salcher *et al.*, 2010). These bacteria appear to be less vulnerable to predation, likely due to specific cell wall properties (Tarao *et al.*, 2009).

Genome analysis (Debroas *et al.*, 2009) and single cell methods (Hornák *et al.*, 2006; Pérez *et al.*, 2009) both suggest that bacteria from the above listed phylogenetic groups (CF, BET, ACT) may prefer distinct components of the dissolved organic matter (DOM) and may even profit from the degradation activity of other taxa (Beier and Bertilson, 2011): The presence of chitin in experimental enrichments specially favoured the growth of free-living ACT that did not seem to be the primary chitin degraders yet readily consumed its hydrolysis product, *N*-acetyl-glucosamine (NAG). Amino sugars generally seem to be an important fraction of lacustrine DOM (Nedoma *et al.*, 1994): Highest NAG concentrations have been found during the spring season in a temperate lake (Hejzlar, 1989), possibly due to its numerous potential sources during a developing algal bloom, such as chitin containing diatoms, algal exopolymeric substances (Blackwel *et al.*, 1967; Giroldo *et al.*, 2003), zooplankton carcasses, faecal pellets (Lee and Fisher, 1992; Tang *et al.*, 2009), and fragments of peptidoglycan released during bacterial growth, by viral lysis, or grazing (Jørgensen *et al.*, 2003; Cloud-Hansen *et al.*, 2006). Hence, NAG seems to be a promising target to explore the competition and niche separation of succeeding phylogenetic groups of freshwater bacteria during this period of the year.

We followed bacterial succession patterns during a spring phytoplankton bloom in an oligomesotrophic lake (Lake Zurich, Switzerland) in the context of abiotic and biotic environmental factors. Furthermore, we investigated the temporal changes of NAG incorporation by the most abundant bacterial groups and compared their NAG uptake patterns with those of a common tracer for bacterial secondary production, leucine (Kirchman *et al.*, 1985). Newly designed oligonucleotide probes were applied to resolve the importance of specific CF lineages, including groups that are not targeted by the general probe CF319a.

Results

Development of the phytoplankton spring bloom

A strong increase of Chl *a* concentration was observed in the epilimnion of Lake Zurich at the beginning of April

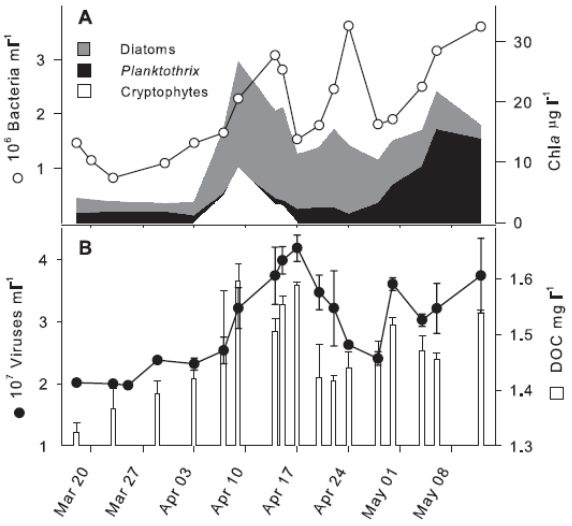

Fig. 1. A. Bacterial cell numbers and total sum of concentrations of diatoms, *P. rubescens* and cryptophytes derived chlorophyll *a* (Chl *a*).
B. Viral cell numbers and concentrations of dissolved organic carbon (DOC). Error bars represent the standard deviation of three samples.

2009 as measured by a multi-wavelength fluoroprobe (Fig. 1A). Total Chl *a* concentrations were also determined by acetone extraction, revealing the same temporal dynamics as by fluormetric measurements; the average difference between the approaches was less than ± 2 µg Chl *a* l^{-1} (data not depicted). The first period of the spring bloom was dominated by cryptophytes (mainly *Rhodomonas* sp., up to 16 µg Chl *a* l^{-1}) and diatoms (up to 18 µg Chl *a* l^{-1}). Towards the end of April these algal groups lost in importance, and the cyanobacterium *Planktothrix rubescens* increasingly dominated the phytoplankton assemblage (Fig. 1A). Details on algal successions as well as on changes in physicochemical parameters are presented in Eugster *et al.* (B. Eugster, E. M. Eckert, F. Pomati, T. Posch and J. Pernthaler, in preparation).

Pronounced fluctuations of total bacterial, viral and nanoflagellate abundances were observed during the study period (Fig. 1). Bacteria reached three times higher numbers after the first peak of Chl *a* than before, of up to 3×10^6 cells ml^{-1}. Both, bacterial and phytoplankton abundances were subsequently strongly reduced, in parallel with a decrease of water temperatures (Fig. 1A, Table S1). A second albeit smaller Chl *a* maximum was followed by another distinct peak of bacterial cell numbers. At the last 3 sampling time points, bacterial abundances steadily increased, in parallel with the rising importance of *P. rubescens*. Dissolved organic carbon (DOC) concentrations were highest during the first peak of the phytoplankton bloom and during its subsequent decline (Fig. 1B). Interestingly, this second maximum of

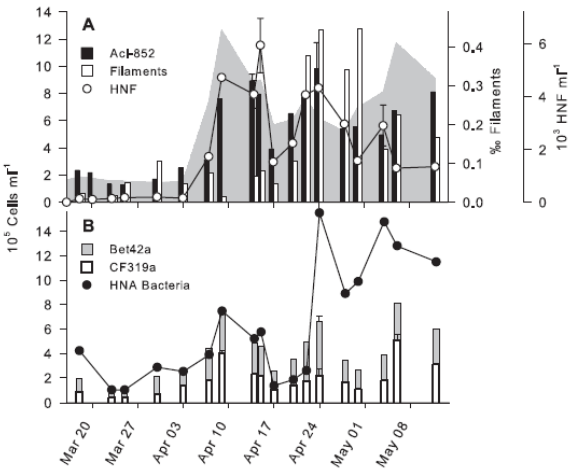

Fig. 2. A. Abundances of Acl *Actinobacteria* (probe Acl-852), heterotrophic nanoflagellates (HNF) and relative abundance of filamentous bacteria. Chlorophyll *a* concentrations (from Fig. 1A, shaded area) are depicted for reference.
B. Abundances of cells with high nucleic acid content (HNA Bacteria), of *Cytophaga–Flavobacteria* (probe CF319a) and *Betaproteobacteria* (probe BET42a). Error bars represent the standard deviation of three samples.

DOC concentrations (as well as two smaller maxima in May) also corresponded to the peak concentrations of viral abundances (Fig. 1B).

Grazing-resistant bacteria

The majority of ACT were affiliated with the Acl subcluster (> 95%, data not shown). Acl bacteria were also the most abundant of all analysed phylogenetic groups during the whole period of elevated Chl *a* concentrations (Fig. 2A). Accounting for approximately 16% of the total bacterioplankton prior to the Chl *a* maximum, Acl bacteria formed up to 36% thereafter (mean 25%), and their total numbers changed by more than 11-fold during the study period. The two distinct maxima of Acl abundances closely matched with the development of non-pigmented heterotrophic nanoflagellates (HNF) (Fig. 2A). A sudden increase of bacteria with filamentous morphologies was observed after the first phytoplankton bloom, and this bloom closely corresponded to the second maximum of HNF abundances (Fig. 2A). Some filamentous bacteria could be identified as *Candidatus* Aquirestis calciphila of *Saprospiraceae*. However, no quantitative analysis of their abundance could be performed due to the pre-filtration of the samples for CARD-FISH staining (data not shown).

Bacteria with high nucleic acid content (HNA) and corresponding phylogenetic groups

The HNA bacteria constituted 33% of total bacterial counts at the first peak of Chl *a*, and substantially less before *and* after this time point (Fig. 2B). Another sudden increase in both, the absolute numbers and the proportions of HNA bacteria was observed after the second peak of Chl *a* on 24 April, and again on 30 April, when they accounted for 43% and 52% of total bacterioplankton cells respectively (Fig. 2B). Bacteria from two major phylogenetic groups that are commonly assigned to the HNA phenotype, CF (as targeted by probe CF 319a) and BET (Šimek *et al.*, 2003), showed pronounced fluctuations throughout the phytoplankton bloom (Fig. 2B). The cell numbers of CF and BET increased approximately 10-fold and eightfold during the phytoplankton bloom, respectively, as compared with the pre-bloom situation. Together they accounted for the majority, if not all, HNA bacteria during the first half of the study period, but only for < 30% in the later samples. CF constituted between 5% and 19% (mean 9%) of total bacterioplankton. They formed three distinct maxima, the first one in parallel to the first Chl *a* peak, and two more on 24 April and 6 May. The temporal dynamics of BET (7–16% of bacterioplankton, mean 10%) mostly matched those of CF; however, these bacteria did not form a peak on 6 May.

Dynamics of specific BET and CF populations

Substantial proportions of BET and CF could be further identified by FISH with specific probes. Bacteria affiliated with the genus *Limnohabitans* (probe R-BT065) accounted for 29–68% (mean 45%) of BET. The maximal abundances of these bacteria mirrored the temporal variability of Chl *a* (Fig. 3). Bacteria affiliated with the type strain *Fluviicola taffensis* and hybridized with probe Flu-736 (Salcher *et al.*, 2011a) accounted for 3–54% (mean 25%) of cells hybridized with probe CF319a (Fig. 3). These bacteria were particularly abundant during

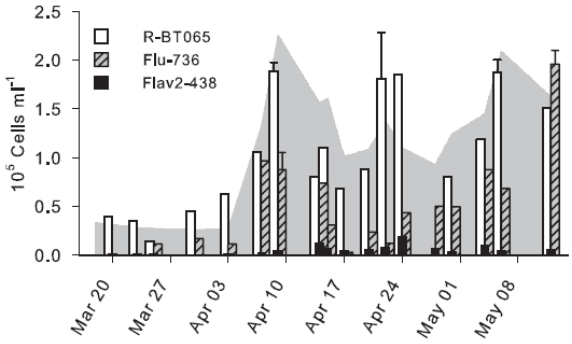

Fig. 3. Abundances of bacteria affiliated with the genus *Limnohabitans* (probe R-BT065) of *Betaproteobacteria*, and bacteria affiliated with *Fluviicola* sp. (probe Flu-736) and the Flav2 clade (probe Flav2-438) of *Cytophaga–Flavobacteria*. Chlorophyll *a* concentrations (from Fig. 1A, shaded area) are depicted for reference. Error bars represent the standard deviation of three samples.

Table 1. Details of the newly designed oligonucleotide probes.

Probe name	Specificity	Target hits (coverage), false positive hits	Sequence (5′ to 3′)	FA %
Cyc715	*Cyclobacteriaceae*	248 (92%), 0	ACAATGCCTWCGCTATCGGTGTT	60
Cyc715C	Competitor for Cyc715		AATATGCCTACGCTATCGGTGTT	
BaVI-C-663	Novel cluster c of baVI[a] lineage	29 (83%), 0	TGTCATATTCCGCCCACAGCA	55
BaVI-C-663-C	Competitor for BaVI-C-663		TGTCATATTCCGCCCACAACA	
BaI-A2-230	Cluster within baI[a] lineage	39 (95%), 4	CTATCTAATCATACGCACACCC	60
BaI-A2-230C1	Competitor for BaI-A2-230		CTASCTAATCATACGCACACCC	
BaI-A2-230C2	Competitor for BaI-A2-230		CTATCTAATCARACGCACACCC	

a. Following the terminology of Newton and colleagues (2011).
FA %, formamide concentration required for the CARD-FISH hybridization buffer.

the first Chl *a* bloom and during the period dominated by *P. rubescens*. Bacteria from the Flav2 lineage that have been found to bloom during a previous spring phytoplankton blooms in Lake Zurich (Zeder *et al.*, 2009), were virtually undetectable before the phytoplankton bloom period, and only reached comparatively low numbers (Fig. 3). Nevertheless, they exhibited clear temporal dynamics, and their highest abundances were observed during the decline of both Chl *a* maxima.

CF clades not targeted by the general probe

Three oligonucleotide probes were developed to determine the importance of bacteria affiliated with CF but not targeted by probe CF319a (Table 1, Fig. 4): Probe Cyc715 targets most members of the family *Cyclobacteriaceae* (CYC), including the uncultured freshwater lineage CL500-6 (baIII-B) (Zwart *et al.*, 2002; Pernthaler *et al.*, 2004; Newton *et al.*, 2011) that also harboured sequence types from our clone libraries (Fig. S1). Probe BaI-A2-230 detects bacteria affiliated with the baI-A2 clade within the family *Chitinophaga* (Newton *et al.*, 2011), i.e. close relatives of the type strain *Sediminibacter salmoneum* (Ou and Yuan, 2008), sequence types obtained in this study and other affiliated sequences. Probe BaVI-C-663 matches to a small cluster of sequences – from various habitats (Fig. S2) – within the baVI lineage of *Sphingobacteria* (Newton *et al.*, 2011) (Fig. 4).

All three probes visualized populations of comparably large and brightly fluorescent cells (Fig. 4). On average CYC constituted 3% of bacterioplankton, and their abundances rose approximately ninefold compared with the pre-bloom situation. During the second half of the bloom (24 April) they accounted for > 10% of all prokaryotes (Fig. 5). Bacteria targeted by probe BaVI-C-663 usually constituted a rather small fraction of all DAPI-stained cells (mean 2%). However, their abundances rose drastically in parallel with the first increase of Chl *a*, when they transiently accounted for more than 6% of all bacteria (Fig. 5). The abundances of bacteria detected with probe BaI-A2-230 never exceeded 2% of all cells and exhibited no pronounced fluctuations during the study period (data not shown).

CF in clone libraries

In total 232 sequences were obtained from clone libraries of water samples from 9, 24 April and 6 May. Of these sequences, 136 were affiliated with CF (66 and 65 in the libraries from 9 and 24 April, respectively, and 5 in the library from 6 May). 110 CF sequences were associated with clades targeted by the probe CF319a, whereas 26 sequences were affiliated with clusters not targeted by this probe. Most of the sequences retrieved on 9 and 24 April were found in clusters of uncultured bacteria most closely related to the type strains *Flavobacterium*

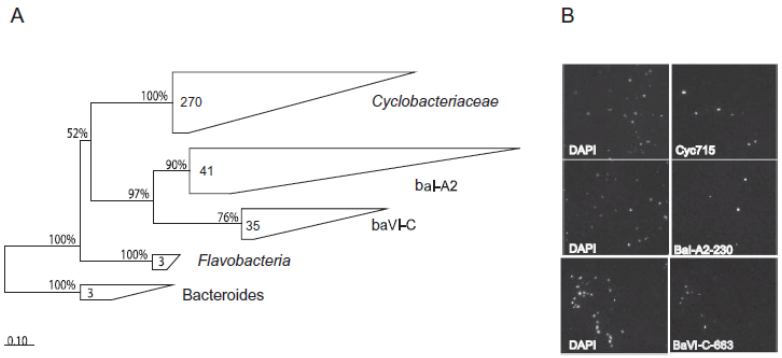

Fig. 4. A. Phylogenetic positioning of the 16S rRNA sequences of *Cyclobacteriaceae*, and the baI-A2 and baVI-C clades of *Sphingobacteriaceae*.
B. Photomicrographs of all bacterioplankton cells (left panel) and the corresponding fractions of bacteria hybridized with the newly designed probes Cyc715, BaI-A2-230 and BaVI-C-663 (right panel). The scale bar (lowest right image) represents 10 µm.

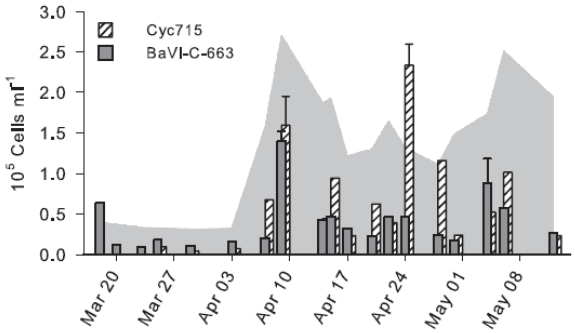

Fig. 5. Abundances of bacteria affiliated with *Cyclobacteriaceae* (probe Cyc715) and the *Sphingobacteria* cluster baVI-C (probe BaVI-C-663). Chlorophyll *a* concentrations (from Fig. 1A, shaded area) are depicted for reference. Error bars represent the standard deviation of three samples.

psychrophilum, *F. terrigena* and *Fluviicola taffensis*. The clone library of 6 May mainly contained sequences from the AC1 cluster of ACT (31 sequences).

Incorporation of N-*acetyl-glucosamine and leucine*

Bulk NAG incorporation rates were considerably lower than of leucine (Fig. 6). NAG uptake ranged from 8–24 pmol l^{-1} h^{-1} (mean 18.4 pmol l^{-1} h^{-1}), whereas community incorporation rates of up to 230 pmol l^{-1} h^{-1} (mean 80 pmol l^{-1} h^{-1}) were observed for leucine. Moreover, the temporal dynamics of leucine and NAG incorporation rates strongly differed: NAG uptake rose during the first phase of the bloom. By contrast, leucine incorporation rates were highest during or immediately after periods of maximal DOC concentrations (Fig. 6 and Fig. 1B). Only 2–7% of all DAPI stained cells (mean 5%) were found to incorporate NAG, whereas 22–43% (mean 29%) of cells incorporated leucine (Fig. 6). Consequently, the mean cell-specific NAG uptake rate (mean 1.6×10^{-19} M $cell^{-1}$ h^{-1}, range $1.2–2.1 \times 10^{-19}$), as calculated from total incorporation rates and proportions of MAR-positive cells matched or even exceeded that of leucine (mean 1.1×10^{-19} M $cell^{-1}$ h^{-1}, range $0.7–1.7 \times 10^{-19}$).

For the assessment of the single cell activity of different taxa the spring bloom period was subdivided into four phases: the first Chl *a* bloom (3–9 April), the subsequent decrease of Chl *a* and peak of DOC (14–17 April), the second, lesser Chl *a* bloom (20–30 April), and the period of increasing dominance of *P. rubescens* (4–12 May) (Fig. 5). The composition of the NAG incorporating fraction of the bacterial community was highly variable over time (Fig. 6, pie charts & Table S2). While CF targeted by probe CF319a and BET dominated NAG uptake in the period of the first Chl *a* bloom (40–43% and 51–57% respectively), ACT accounted for the major fraction (41–61%) in the subsequent phase. CYC targeted with probe Cyc715 constituted up to 15% of the NAG-active community during the second bloom, and their contribution was in the range of BET and ACT (both 16%). A large fraction of the NAG incorporating bacteria in this period remained unidentified (46%). With rising importance of *P. rubescens* in the phytoplankton community, the NAG-active assemblage was again dominated by ACT (14–61%, mean 33%), whereas the contribution of all other phylogenetic groups was rather similar (BET, 8–13%; CF, 13–26%;

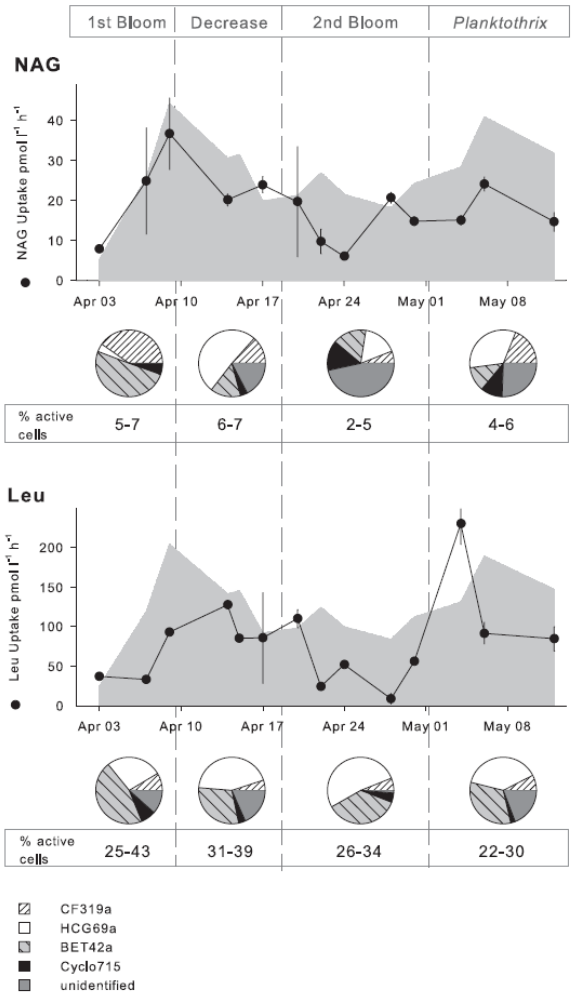

Fig. 6. Graphs: Total community uptake of *N*-acetyl-glucosamine (NAG) and Leucine (Leu). Chlorophyll *a* concentrations (from Fig. 1A, shaded area) are depicted for reference. Pie charts: composition of the active community as determined by MAR-FISH with probes CF319a, HGC69a (*Actinobacteria*), BET42a and Cyc715 (mean values of 3–5 time points per period). The numbers below represent the percentage of the total bacterial community incorporating the substrate. Each chart represents average data from one of four periods (1st bloom: 3–9 April, Decrease: 14–17 April, 2nd bloom: 20–30 April, *Planktothrix*: 4–12 May). Error bars represent the standard deviation of three samples.

CYC, 4–18%). No bacteria from the CF clades baI-A2 (*Sediminibacter*) and baVI (*Sphingobacteria*) were observed to incorporate NAG.

The leucine-active community comprised 15–66% ACT, 21–60% BET, 6–10% CF hybridized with CF319a and 2–7% CYC, leaving a fraction of 2–19% unidentified active cells. BET constituted the major fraction of active cells (35–60%) during the first Chl *a* bloom, whereas ACT dominated the leucine-active assemblage during the two subsequent periods (38–66%). During the final period BET and ACT contributed approximately equally to leucine incorporation (25–38% and 20–56% respectively). While 96–100% of cells from the R-BT065 (*Limnohabitans*) clade showed high leucine incorporation rates, no NAG uptake was observed in these bacteria (data not depicted). Bacteria hybridized with the probe Flu-736 did not incorporate either tracer (data not depicted). During the first bloom period, the proportion of NAG-incorporating cells from the Flav2 clade (22%) was almost twice as high as the proportions of NAG-incorporating CF in general (10–13%) (data not depicted). In contrast, Flav2 bacteria did not visibly incorporate leucine.

Discussion

Factors influencing the bacterial community

Phytoplankton spring blooms are characterized by alternating dominance of bottom-up and top-down control on the heterotrophic bacterial assemblages (Weisse *et al.*, 1990). This interplay is also reflected in the development of the bacterioplankton in Lake Zurich during the first two consecutive phytoplankton blooms (Fig. 1): Neither changes in DOC nor mortality-related factors such as the abundances of HNF or viruses could by themselves predict the dynamics of the bacterial community during these periods (as reflected by low and non-significant coefficients of pairwise Pearson product-moment correlations). Bacterial numbers and Chl *a* concentrations were significantly correlated over the whole study period (Pearson correlation coefficient $r = 0.79$, $P_{adjusted} < 0.001$), but there were also conspicuous discrepancies in the time-courses of these parameters. For example, while the first peak of Chl *a* was much more pronounced, total bacterial numbers only reached a maximum after the second, much smaller, phytoplankton bloom (Fig. 1A). This rapid bacterial growth might have been stimulated by the additional input of DOC at that time point, which, in turn, was possibly related to the presumably enhanced lysis of picoplankton and/or algal cells during the maximum period of viral abundances (Fig. 1B, Chl *a* versus DOC: $r = 0.66$, $P_{adjusted} < 0.05$; viruses versus DOC: $r = 0.79$, $P_{adjusted} < 0.001$).

Viruses are regarded as mediators between bottom-up and top-down control, since they influence bacterial communities both, as agents of mortality and as producers of DOC (Middelboe and Jørgensen, 2006). Changes of viral and bacterial abundances in lakes are often closely related, and virus-like particles may be particularly abundant during spring (Personnic *et al.*, 2009; Ram *et al.*, 2010). A similar dual influence is also ascribed to HNF (Nagata and Kirchman, 1992) due to their recycling of limiting nutrients (Goldman and Caron, 1985). In addition, they are also promoters of genotypic successions in freshwater bacterioplankton (Jürgens *et al.*, 1999): during the first phase of the bloom the increasing importance of HNF was accompanied by a shift from typically fast-growing bacterioplankton taxa such as CF and BET (Jürgens *et al.*, 1999) towards more grazing resistant Ac1 ACT (Pernthaler *et al.*, 2001) (Fig. 2). HNF abundances peaked a second time during the later stage of the bloom, which presumably favoured the rise of additional groups of grazing resistant bacteria, i.e. of filamentous morphotypes (Jürgens and Sala, 2000; Pernthaler *et al.*, 2004) (Fig. 2A).

Dynamics of specific populations

Each of the analysed bacterial population exhibited a unique pattern of abundance changes (Figs 3 and 5), altogether representing a succession of asynchronous blooms throughout the spring period. For example, the dynamics of 'opportunistically' growing (Šimek *et al.*, 2005) typical freshwater BET affiliated with *Limnohabitans* (as targeted by probe R-BT065) clearly differed from that of *Fluviicola*-like bacteria hybridized with probe Flu-736 (Fig. 3). The latter group formed up to 1.9×10^5 cells ml^{-1}, and thus reached similarly high abundances as *Limnohabitans* spp. (Fig. 3) that are among the most important members of lake bacterioplankton (Šimek *et al.*, 2005).

Fluviicola-like bacteria represented up to 62% of all CF (Figs 2B and 3). CF in pelagic environments are highly diversified (Alonso *et al.*, 2007; Eiler and Bertilsson, 2007) and numerous small, specialized populations affiliated with this phylum are believed to coexist in the bacterioplankton (Eiler and Bertilsson, 2007). To our knowledge, this is the first time that the majority of freshwater CF could be assigned to a single genus-like taxon. Moreover, the *Fluviicola*-like bacteria strongly increased in abundance with rising importance of *P. rubescens* (Figs 1 and 3). This might allow novel insight into their specific ecological niche: The probe Flu-736 was originally designed to target rRNA gene sequences obtained from Lake Zurich during autumn that were only present in water layers with the highest densities of *P. rubescens* (Van den Wyngaert *et al.*, 2011; Salcher *et al.*, 2011a). However, bacteria hybridized with Flu-736 only constituted around 10% of CF and below 1% of total bacteria in those samples. Moreover, they only occurred together with

P. rubescens and were entirely absent in other water layers. It is, therefore, conceivable that Fluviicola-like bacteria in Lake Zurich are closely associated with blooms of primary producers, and that the *P. rubescens* dominated water layers may provide them with a 'refuge' between such events.

CF not targeted by probe CF319a

Our results illustrate the potential importance of CF that are not hybridized with probe CF319a in freshwater bacterioplankton: Members of the baVI-C cluster and of the family *Cyclobacteriaceae* together constituted up to > 10% of the total bacterial assemblage (Fig. 5) in Lake Zurich. Bacteria hybridized with probe BaVI-C-663 represent a novel, phylogenetically narrow lineage within the recently defined baVI cluster of CF (Newton *et al.*, 2011) (Fig. S2). Besides genotypes from freshwaters, this clade harbours rRNA gene sequences from various other habitats (Fig. S2), as is also the case for other lineages of the baVI group (Newton *et al.*, 2011). Fluctuations of environmental parameters seemed to allow for transient blooms of these 'opportunistically' growing bacteria (with maximal apparent doubling times of < 12h) (Fig. 5) that were possibly terminated by top-down control. Moreover, the close phylogenetic relationship of baVI-C genotypes obtained from freshwaters with others from soil and activated sludge suggests that these bacteria need not necessarily be members of the free-living microbial assemblage only but might also be able to colonize suspended organic aggregates, i.e. 'lake snow' formed by senescent algal cells during the later stages of phytoplankton blooms (Kiørboe *et al.*, 2002).

Bacteria affiliated with the family *Cyclobacteriaceae* were remarkably abundant during the phytoplankton bloom, reaching similarly high numbers as cells hybridized with the FISH probes CF319a and Bet42a (Figs 2B and 5). Most type-strains of the family *Cyclobacteriaceae* are associated with marine or freshwater habitats (see Kumar *et al.*, 2010b and references therein), and almost 50% (79 of 163) of the *Cyclobacteriaceae* in the SILVA rRNA gene reference database (release 102; Pruesse *et al.*, 2007) originate from isolates. This may suggest that *Cyclobacteriaceae* are rather underrepresented in clone libraries from freshwater systems. The high cultivability of freshwater *Cyclobacteriaceae* on solid media might moreover hint at their ability to colonize suspended organic particles in lake water. However, while many representatives from this family can be isolated, the rRNA gene sequences obtained in this study are affiliated with a lineage of so far uncultured freshwater and estuarine bacteria within the genus *Algoriphagus* (Nedashkovskaya *et al.*, 2007) (Fig. S1). This clade has been described as the typical freshwater group CL500-6 by Zwart and colleagues (2002) and has recently been renamed to baIII-B (Newton *et al.*, 2011). Closely related sequence types have been previously found in a mesotrophic lake during the decline of the algal spring bloom (Pernthaler *et al.*, 2004), and in oligotrophic high altitude lakes with high abundances of the cyanobacterium *Synechococcus* (Xing *et al.*, 2009) (Fig. S1). This may indicate a possibly tight connection of baIII-B bacteria with the phytoplankton.

Substrate incorporation

We analysed the uptake of two different, albeit stoichiometrically similar low-molecular-weight substrates that are components of both, the carbon and the nitrogen cycle of aquatic habitats: NAG ($C_8H_{15}NO_6$) and leucine ($C_6H_3NO_2$). The ambient concentrations of amino sugars and amino acids have been reported to be in the same order of magnitude in aquatic ecosystems (Berman and Bronk, 2003). Nevertheless, the incorporation rates of NAG during the spring phytoplankton bloom in Lake Zurich were considerably lower than of leucine (Fig. 6). This agrees with results from marine pelagic habitats (Zubkov *et al.*, 2008): in that study the concentrations of dissolved NAG and glucose were found to exceed those of amino acids, whereas highest bacterial uptake was observed for leucine. Amino acids account for over 50% of the carbon content of pelagic bacteria (Simon and Azam, 1989); it is hypothesized that cells might save energy by specializing on amino acid utilization and thereby circumventing their *de novo* biosynthesis (Kirchman, 1990). However, the bacterial incorporation of amino acids or carbohydrates in lakes might vary throughout the year, with a clear preference for amino acids during spring phytoplankton blooms (Weiss and Simon, 1999).

A remarkably smaller fraction of cells were responsible for the uptake of NAG than of leucine (Fig. 6). This implies that the average incorporation rate of both substrates per bacterial cell was in a similar range. Thus, NAG-incorporation was not a general, subsidiary activity of the growing fraction of the bacterioplankton, but was rather performed by populations of highly effective specialists. Moreover, while the taxonomic composition of leucine-active bacteria was rather constant, a pronounced successional pattern was observed within the NAG-active bacteria (Fig. 6): CF and BET dominate NAG uptake during the initial phase of rapid algal growth (Fig. 1A), whereas ACT constituted the major fraction during the subsequent period of high HNF densities (Fig. 2A). This shift was reflected both, in the respective contributions of the different groups to the NAG-active community and in the proportions of active cells within these groups (data not shown). CYC constituted an important fraction of NAG incorporating cells only in the third period of the spring

bloom (after the decline of cryptophytes, Fig. 1A), and their higher contribution to the NAG-active community was paralleled by their rising abundances. The ability to grow on NAG seems to be widespread within this group, because 13 of 16 tested *Algoriphagus* type-strains were capable of NAG utilization (Young *et al.*, 2009).

Only up to approximately 10% of CF, BET, CYC and ACT were found to incorporate NAG. By contrast, the NAG-active fraction of the generally rare Flav2 bacteria (Fig. 3) was about twice as high (data not shown). An isolate from this lineage (Zeder *et al.*, 2009) was found capable of growing on NAG as the sole carbon source (E. Eckert, unpubl. data). These bacteria seem to follow an 'opportunistic' life strategy in that they are capable of very high growth rates, but are likely suppressed by flagellate grazing (Zeder *et al.*, 2009). Thus the turnover of some substrates due to such small populations could be much higher than predicted by their standing stocks.

The involvement of all major lineages of limnetic bacteria in NAG uptake *in situ* is in good agreement with recent observations from chitin enrichment experiments (Beier and Bertilson, 2011). Our results extend these findings by showing that the contrasting roles of CF and ACT in NAG turnover (Beier and Bertilson, 2011) may not only be due to their metabolic differences but might also be related to characteristics of the food web. Grazer-mediated shifts in bacterial activity have been described before (Jürgens and Sala, 2000), and the genotypic changes of NAG-active bacteria (Fig. 6) might be – directly or indirectly – related to their differential vulnerability to predation. For one, it is possible that ACT were less competitive in NAG uptake and directly profited from the reduction of competitors. Individual taxa within the bacterioplankton are known to differ in substrate affinity, i.e. in their concentration dependent uptake patterns (Alonso and Pernthaler, 2006; Salcher *et al.*, 2011b). On the other hand, the observed successions within the NAG-active community might have also resulted from changing sources, turnover or concentrations of this substrate: Kawasaki and Benner (2006) showed an increase of dissolved NAG in marine microcosms in parallel with the increasing senescence of the microbial community, which in fact might have been related to the release and subsequent hydrolysis of incompletely digested cellular material during flagellate grazing (Nagata and Kirchman, 1992). It is thus intriguing to speculate that the comparatively small subpopulation of the highly NAG-active and presumably grazing-protected (Tarao *et al.*, 2009) ACT in our samples might in fact be specialized for the uptake of a substrate that in parts originated from the foraging of HNF on other bacterial taxa (e.g. on CF or BET). In any case, it appears conceivable that the various growth strategies of bacterial populations involved in the uptake of a particular DOM component (such as NAG) might eventually decide whether aquatic microbial assemblages act as links or sinks (Sherr *et al.*, 1987) for this component.

Experimental procedures

Sampling site and sample collection

Lake Zurich is an oligomesotrophic pre-alpine lake (max. depth; 136 m, surface area; 66.8 km^2) with a theoretical water renewal time of 1.2 years. The lake is monomictic but only infrequently holomictic (Bossard *et al.*, 2001). Sampling on Lake Zurich was conducted two to three times a week during the period of the spring phytoplankton bloom, from 13 March through 12 May 2009 at approximately 10 a.m and coordinates N47 19.3 E8 33.9. Chl *a* concentrations were determined between 0 and 40 m depth using a multiple wavelength probe (TS-16–12 fluoroprobe, bbe Moldaenke GmbH, Kronshagen, Germany). Depth profiles of temperature were determined with a multi-parameter probe (6600 multi-parameter, water quality monitoring, YSI incorp., Yellow Springs, OH, USA). The vertical Chl *a* distribution patterns were analysed on the boat prior to the collection of water samples. Depending on these measurements, water was collected from the depth of the maximum Chl *a* concentration. Two litres of water were filled into glass bottles (Schott) and transported to the laboratory in closed isolated boxes within 30 min. Samples were pre-filtered (pore size, 0.8 µm, Millipore, Billerica, MA, USA) to exclude algae and particle-bound microbes. Subsamples without prior pre-filtration were used to determine the abundances of filamentous bacterial morphotypes (see below). Table S1 summarizes the sampling dates, depths and corresponding water temperatures.

Samples for the determination of total cell numbers, of filamentous bacteria, and of the proportions of different bacterial taxa were fixed with buffered paraformaldehyde fixative (PFA, 1% w/v). Samples for counting viral abundances were fixed with ice-cold glutaraldehyde (0.5% w/v) at 4°C for 30 min, frozen in liquid nitrogen and stored at −80°C (Brussaard, 2004). Portions of 4–5 ml of PFA-fixed samples were filtered onto white polycarbonate membrane filters (type GTTP, 45 mm diameter, 0.2 µm pore size, Millipore) and stored at −20°C for subsequent community analysis by fluorescence *in situ* hybridization and catalysed reporter deposition (CARD-FISH). Unfixed samples (200 ml) were filtered onto polysulfone filters (type GPWP, diameter, 47 mm, pore size, 0.22 µm, Millipore) for later DNA extraction and 16S rRNA gene sequencing, and stored at −80°C until further processing.

Chl a *analysis*

A multiple wavelength probe (TS-16–12 fluoroprobe, bbe Moldaenke GmbH, Kronshagen, Germany) was used to measure Chl *a* and pigment concentrations at six different wavelengths (370, 470, 525, 570, 590, 610 nm). This probe was calibrated to distinguish between different phytoplankton groups based on their respective patterns of pigment fluorescence based on which the corresponding software calculates the contribution of different algal groups to the total Chl *a*. In order to confirm probe performance, total Chl *a* was additionally also determined by acetone extraction as described by Lorenzen (1967).

Organic carbon analysis

Total organic carbon (TOC) was determined on unfiltered water samples and DOC on pre-rinsed nitrocellulose membrane filter (NC 20, 0.2 μm, 50 mm, Schleicher und Schuell, Dassel, Germany) using a TOC analyser (TOC-5000, Shimadzu, Kyoto, Japan) according to the manufacturer's instructions.

Bacterial and viral abundances

Total bacterial abundances were determined by flow cytometry (inFlux V-GS, Becton Dickinson, Franklin Lakes, NJ, USA). One millilitre of PFA fixed samples was stained with 10 μl of 4′,6-diamidino-2-phenylindole (DAPI, 1 μg ml^{-1} final concentration). A UV laser (60 mW, 355 nm) served as excitation source and DAPI emission was measured at 460 ± 50 nm. Side scatter (SSC) was determined with a blue laser (488 nm). Scatter Bi-plots of DNA fluorescence and SSC were analysed in FlowJo 7.1.2 (Tree star, Ashland, OR, USA). In addition to total cell numbers, bacteria with high and low nucleic acid content (HNA, LNA) were distinguished. Viral abundances were determined as described by Brussaard (2004). The total numbers of filamentous bacterial morphotypes were determined microscopically on a Zeiss AxioImager M (Carl Zeiss, Jena, Germany) after DAPI staining and filtration (2 ml) on black polycarbonate filters (0.2 μm pore size, GE Osmonics, Minnetonka, MN, USA). Two thousand to 10000 DAPI stained cells and 30–100 filaments were counted per sample. Heterotrophic flagellates were counted using an epifluorecence microscope (at least 50 flagellates per sample).

CARD-FISH analysis was carried out following the protocol of Sekar and colleagues (2003), with minor modifications. Changes to this protocol were: hybridization periods of 2–4 h, washing for 30 min, a second washing step of 45 min in 1 × phosphate-buffered saline with 0.01% Triton X-100 (PBS-T) at 37°C to reduce background, and signal amplification for 30 min. The oligonucleotide probes (including corresponding helpers and competitors) used for CARD-FISH analysis were CF319a for CF (Manz *et al.*, 1996), LD2-739 for bacteria affiliated with *Candidatus* Aquirestis calciphila (Pernthaler *et al.*, 2004), Flav2-438 for the Flav2 subcluster (Zeder *et al.*, 2009), Flu-736 for *Fluviicola*-like bacteria (Salcher *et al.*, 2011a), Bet42a for BET (Manz *et al.*, 1992) and R-BT065 for the *Limnohabitans* genus (Šimek *et al.*, 2001b), HGC69a for ACT (Roller *et al.*, 1994) and AC1-852 for the AC1 lineage (Warnecke *et al.*, 2005). For probes designed in this study see below and Table 1.

Substrate incorporation

Samples from each time point were incubated with [^{3}H]-*N*-acetyl-D-glucosamine (specific activity 9 Ci $mmol^{-1}$) and [^{3}H]-leucine (specific activity 62 Ci $mmol^{-1}$, GE Healthcare, Glattbrugg, Switzerland) in order to determine bulk community incorporation rates and the substrate uptake by different taxa via micro-autoradiography and CARD-FISH (MAR-FISH). Five replicate portions of 10 ml were placed in 15 ml tubes, and two of the samples (controls) were immediately fixed with PFA (w/v 1%). Tracer was then added at a concentration of 10 nM, and the tubes were incubated in the dark at *in situ* temperature (Table S1). After 2 h of incubation samples were fixed with PFA (w/v 1%) for 45 min at room temperature.

Five millilitres from each tube was filtered onto nitrocellulose filters (25 mm, pore size 0.22 μm, Millipore) and washed twice with 3 ml of ice-cold Trichloroacid and with ice-cold 80% ethanol (Kirchman *et al.*, 1985). Measurements of incorporated radioactivity were conducted on a scintillation counter (Tri-Carb 3170TR/SL, PerkinElmer, Waltham, MA, USA). The remaining 3 × 5 ml of radiolabelled samples (or 2 × 5 ml of pre-fixed controls) were pooled into single tubes. Five millilitres from these pooled samples was reduced to a volume of 1 ml by filtration onto a GTTP filter (25 mm, pore size 0.2 μm, Millipore) at low pressure (< 200 mmHg). Two millilitres of sterile deionized water was added, and the total volume was again reduced to 1 ml. This step was repeated 1–2 times. Cells were resuspended by repeated pumping with a pipette and the concentrated cell suspension was transferred to a 1.5 ml reaction vial. Subsequently, 25 μl of this solution was spotted onto glass coverslips (24 × 60 mm). Each coverslip held spotted samples from 2–3 dates that had been incubated with the same tracer. The spots on the coverslips were marked using a PAP–pen (Liquid Blocker, Sigma-Aldrich, St. Louis, MO, USA) and dried at 46°C for approximately 1 h. Subsequently each spot was covered with 25 μl of a 0.01% low melting point agarose solution (BioConcept, Allschwil, Switzerland). The coverslips were dried at 46°C and then stored at −20°C until further processing. To ensure that no bias of community composition was introduced by the cell concentration procedure, the relative abundances of hybridized cells in samples concentrated on coverslips were compared with those of filtered samples. This was tested for *Bacteria* (probe EUBI-III), Beta (probe Bet42a) and Act (probe HGC69a). No significant differences between the two approaches could be observed for either probe (data not shown).

Microautoradiography

The coverslips with concentrated bacterial cells were first attached to microscopic slides and fixed in place with parafilm. To facilitate their later detachment (after development), a thin layer of immersion oil was applied between the coverslip and the slide. MAR-FISH was then performed as previously described (Alonso and Pernthaler, 2005). The exposure time of the photographic emulsion varied between 2–4 days for leucine and 6–10 days for NAG.

Microscopic evaluation

CARD-FISH and MAR-FISH samples were evaluated using a fully automated microscopic platform (AxioImager Z1) (Zeder and Pernthaler, 2009). Since cells were often unevenly distributed, multi-spot autofocus (Zeder and Pernthaler, 2009) was disabled for imaging of MAR-FISH samples to allow a better localization of the cell layer. Image quality control was conducted using an artificial neural network which had been trained on one of the spring bloom image sets (Zeder *et al.*, 2010). A minimum of 10 high quality images and 8000 DAPI

stained cells were evaluated per sample and probe. Abundance and substrate incorporation small bacterial populations (Flav2, baVI-C, baI-A2) were determined by manual counting (at least 1000 DAPI stained cells and 10 fields for abundance, and 150 hybridized cells and 10 fields for substrate incorporation).

Cloning and 16S rRNA analysis

Clone libraries of 16S rRNA genes were constructed from three time points: 9, 24 April and 6 May. DNA extraction, PCR, purification and sequencing were conducted as described in Shabarova and Pernthaler (2010). The purified PCR products were cloned into competent *Escherichia coli* (pGEM-T® Easy Vectors, Promega Corporation, Madison WI, USA). Insert harbouring plasmids were detected using PCR (GoTaq® Green Master Mix, Promega) and purified with GenElute™ Five-Minute Plasmid Miniprep Kits (Sigma-Aldrich). Almost complete 16S rRNA gene sequences were assembled from several sequencing runs using DNA Baser Sequence Assembler (Heracle BioSoft S.R.L., Romania).

Phylogenetic analysis and FISH probe design

Sequences were aligned using the SINA web aligner (Pruesse *et al*., 2007). The alignments were merged into the SILVA SSU reference database release 102 using the ARB software package (Ludwig *et al*., 2004). Sequences were deposited to the EMBL database with accession numbers HE574166 to HE574397. Only high quality sequences $>$ 1000 nucleotides and with a Pintail value $>$ 90 were used for phylogenetic analysis (Ashelford *et al*., 2005). The closest cultivated relatives were selected from the 'all species living tree' project (Yarza *et al*., 2008). Bootstrapped maximum likelihood trees (100 repetitions) were calculated with sequences affiliated with the groups of interest and close relatives on a dedicated web server (Stamatakis *et al*., 2008).

The ARB probe design tool was used to develop oligonucleotide probes for three clusters not hybridized with the probe CF319a: a cluster affiliated with *Sphingobacteria* (BaVI-C-663), the family *Cyclobacteriaceae* (Cyc715), and a cluster affiliated with *Sediminibacter* sp. (BaI-A2-233) (Table 1). The theoretical coverage of the probe was evaluated *in silico* against the SILVA SSU reference database (release 102). In order to define stringent conditions for CARD-FISH, extensive tests on environmental samples were performed using formamide concentrations from 25–70% in the hybridization buffer (Table 1).

Statistical analysis

Pearson correlations between Chl *a*, DOC, viral, bacterial and flagellate abundances were conducted in SigmaPlot statistic pack, version 11 (Systat Software). Significance levels of all correlations were adjusted to account for multiple testing (Bonferroni method).

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References

Abell, G.C.J., and Bowman, J.P. (2005) Colonization and community dynamics of class *Flavobacteria* on diatom detritus in experimental mesocosms based on Southern Ocean seawater. *FEMS Microbiol Ecol* **53:** 379–391.

Alonso, C., and Pernthaler, J. (2005) Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. *Appl Environ Microbiol* **71:** 1709–1716.

Alonso, C., and Pernthaler, J. (2006) Concentration-dependent patterns of leucine incorporation by coastal picoplankton. *Appl Environ Microbiol* **72:** 2141–2147.

Alonso, C., Warnecke, F., Amann, R., and Pernthaler, J. (2007) High local and global diversity of *Flavobacteria* in marine plankton. *Environ Microbiol* **9:** 1253–1266.

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., and Weightman, A.J. (2005) At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Appl Environ Microbiol* **71:** 7724–7736.

Beier, S., and Bertilson, S. (2011) Uncoupling of chitinase activity and uptake of hydrolysis products in freshwater bacterioplankton. *Limnol Oceanogr* **56:** 1179–1188.

Berman, T., and Bronk, D.A. (2003) Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquat Microb Ecol* **31:** 279–305.

Biddanda, B.A., and Cotner, J.B. (2002) Love handles in aquatic ecosystems: the role of dissolved organic carbon drawdown, resuspended sediments, and terrigenous inputs in the carbon balance of Lake Michigan. *Ecosystems* **5:** 431–445.

Blackwel, J., Parker, K.D., and Rudall, K.M. (1967) Chitin fibers of diatoms *Thalassiosira fluviatilis* and *Cyclotella cryptica*. *J Mol Biol* **28:** 383–385.

Bossard, P., Gammeter, S., Lehmann, C., Schanz, F., Bachofen, R., Burgi, H.R., *et al.* (2001) Limnological description of the Lakes Zurich, Lucerne, and Cadagno. *Aquat Sci* **63:** 225–249.

Brussaard, C.P.D. (2004) Optimization of procedures for counting viruses by flow cytometry. *Appl Environ Microbiol* **70:** 1506–1513.

Cloud-Hansen, K.A., Peterson, S.B., Stabb, E.V., Goldman, W.E., McFall-Ngai, M.J., and Handelsman, J. (2006) Breaching the great wall: peptidoglycan and microbial interactions. *Nat Rev Microbiol* **4:** 710–716.

Debroas, D., Humbert, J.F., Enault, F., Bronner, G., Faubladier, M., and Cornillot, E. (2009) Metagenomic approach studying the taxonomic and functional diversity of the bacterial community in a mesotrophic lake (Lac du Bourget – France). *Environ Microbiol* **11:** 2412–2424.

Eiler, A., and Bertilsson, S. (2007) *Flavobacteria* blooms in four eutrophic lakes: linking population dynamics of

freshwater bacterioplankton to resource availability. *Appl Environ Microbiol* **73:** 3511–3518.

Fandino, L.B., Riemann, L., Steward, G.F., and Azam, F. (2005) Population dynamics of *Cytophaga–Flavobacteria* during marine phytoplankton blooms analyzed by real-time quantitative PCR. *Aquat Microb Ecol* **40:** 251–257.

Giroldo, D., Vieira, A.A.H., and Paulsen, B.S. (2003) Relative increase of deoxy sugars during microbial degradation of an extracellular polysaccharide released by a tropical freshwater *Thalassiosira* sp. (*Bacillariophyceae*). *J Phycol* **39:** 1109–1115.

Goldman, J.C., and Caron, D.A. (1985) Experimental studies on an omnivorous microflagellate – implications for grazing and nutrient regeneration in the marine microbial food-chain. *Deep Sea Res A* **32:** 899–915.

Gómez-Pereira, P.R., Fuchs, B.M., Alonso, C., Oliver, M.J., van Beusekom, J.E.E., and Amann, R. (2010) Distinct *flavobacteria*l communities in contrasting water masses of the North Atlantic Ocean. *ISME J* **4:** 472–487.

Grossart, H.P., Levold, F., Allgaier, M., Simon, M., and Brinkhoff, T. (2005) Marine diatom species harbour distinct bacterial communities. *Environ Microbiol* **7:** 860–873.

Hejzlar, J. (1989) Dissolved amino sugars in the Rimov Reservoir (Czechoslovakia). *Ergeb Limnol* **33:** 291–301.

Hornák, K., Jezbera, J., Nedoma, J., Gasol, J.M., and Šimek, K. (2006) Effects of resource availability and bacterivory on leucine incorporation in different groups of freshwater bacterioplankton, assessed using microautoradiography. *Aquat Microb Ecol* **45:** 277–289.

Jezbera, J., Hornák, K., and Šimek, K. (2006) Prey selectivity of bacterivorous protists in different size fractions of reservoir water amended with nutrients. *Environ Microbiol* **8:** 1330–1339.

Jones, S.E., Newton, R.J., and McMahon, K.D. (2009) Evidence for structuring of bacterial community composition by organic carbon source in temperate lakes. *Environ Microbiol* **11:** 2463–2472.

Jørgensen, N.O.G., Stepanaukas, R., Pedersen, A.G.U., Hansen, M., and Nybroe, O. (2003) Occurrence and degradation of peptidoglycan in aquatic environments. *FEMS Microbiol Ecol* **46:** 269–280.

Jürgens, K., and Sala, M.M. (2000) Predation-mediated shifts in size distribution of microbial biomass and activity during detritus decomposition. *Oikos* **91:** 29–40.

Jürgens, K., Pernthaler, J., Schalla, S., and Amann, R. (1999) Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. *Appl Environ Microbiol* **65:** 1241–1250.

Kawasaki, N., and Benner, R. (2006) Bacterial release of dissolved organic matter during cell growth and decline: molecular origin and composition. *Limnol Oceanogr* **51:** 2170–2180.

Kiørboe, T., Grossart, H.P., Ploug, H., and Tang, K. (2002) Mechanisms and rates of bacterial colonization of sinking aggregates. *Appl Environ Microbiol* **68:** 3996–4006.

Kirchman, D., Knees, E., and Hodson, R. (1985) Leucine incorporation and its potenial as a measure of protein-sythesis by bacteria in natural aquatic systems. *Appl Environ Microbiol* **49:** 599–607.

Kirchman, D.L. (1990) Limitation of bacterial growth by dissolved organic matter in the sub arctic Pacific. *Mar Ecol Prog Ser* **62:** 47–54.

Kumar, P.A., Srinivas, T.N.R., Kumar, P.P., Madhu, S., and Shivaji, S. (2010a) *Nitritalea halalkaliphila* gen. nov., sp. nov., an alkaliphilic bacterium of the family '*Cyclobacteriaceae*', phylum *Bacteroidetes*. *Int J Syst Evol Microbiol* **60:** 2320–2325.

Kumar, P.A., Srinivas, T.N.R., Madhu, S., Manorama, R., and Shivaji, S. (2010b) *Indibacter alkaliphilus* gen. nov., sp. nov., an alkaliphilic bacterium isolated from a haloalkaline lake. *Int J Syst Evol Microbiol* **60:** 721–726.

Lee, B.G., and Fisher, N.S. (1992) Decomposition and release of elements from zooplankton debris. *Mar Ecol Prog Ser* **88:** 117–128.

Lorenzen, C.J. (1967) Determination of chlorophyll and pheopigments: spectrophotometric equations. *Limnol Oceanogr* **12:** 343–346.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363–1371.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria* – problems and solutions. *Syst Appl Microbiol* **15:** 593–600.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., and Schleifer, K.H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology* **142:** 1097–1106.

Middelboe, M., and Jørgensen, N.O.G. (2006) Viral lysis of bacteria: an important source of dissolved amino acids and cell wall compounds. *J Mar Biol Assoc UK* **86:** 605–612.

Nagata, T., and Kirchman, D.L. (1992) Release of macromolecular organic-complexes by heterotrophic marine flagellates. *Mar Ecol Prog Ser* **83:** 233–240.

Nedashkovskaya, O.I., Kim, S.B., Kwon, K.K., Shin, D.S., Luo, X., Kim, S.J., and Mikhailov, V.V. (2007) Proposal of *Algoriphagus vanfongensis* sp. nov., transfer of members of the genera *Hongiella* Yi and Chun 2004 emend. Nedashkovaskaya *et al.* 2004 and *Chimaereicella* Tiago *et al.* 2006 to the genus *Algoriphagus*, and emended description of the genus *Algoriphagus* Bowman *et al.* 2003 emend. Nedashkovaskaya *et al.* 2004. *Int J Syst Evol Microbiol* **57:** 1988–1994.

Nedoma, J., Vrba, J., Hejzlar, J., Šimek, K., and Straskrabová, V. (1994) *N*-acetylglucosamine dynamics in freshwarer environments – concentration of amino-sugars, extracellular enzyme-activities, and microbial uptake. *Limnol Oceanogr* **39:** 1088–1100.

Newton, R.J., Jones, S.E., Eiler, A., McMahon, K.D., and Bertilsson, S. (2011) A guide to the natural history of freshwater lake bacteria. *Microbiol Mol Biol Rev* **75:** 14–49.

Ou, J.H., and Yuan, H.L. (2008) *Sediminibacterium salmoneum* gen. nov., sp. nov., a member of the phylum *Bacteroidetes* isolated from sediment of a eutrophic reservoir. *Int J Syst Evol Microbiol* **58:** 2191–2194.

Pérez, M.T., Hörtnagl, P., and Sommaruga, R. (2009) Contrasting ability to take up leucine and thymidine among freshwater bacterial groups: implications for bacterial production measurements. *Environ Microbiol* **12:** 74–82.

Pernthaler, J., Posch, T., Šimek, K., Vrba, J., Pernthaler, A., Glöckner, F.O., *et al.* (2001) Predator-specific enrichment of *Actinobacteria* from a cosmopolitan freshwater clade in mixed continuous culture. *Appl Environ Microbiol* **67:** 2145–2155.

Pernthaler, J., Zöllner, E., Warnecke, F., and Jürgens, K. (2004) Bloom of filamentous bacteria in a mesotrophic lake: Identity and potential controlling mechanism. *Appl Environ Microbiol* **70:** 6272–6281.

Personnic, S., Domaizon, I., Sime-Ngando, T., and Jacquet, S. (2009) Seasonal variations of microbial abundances and virus- versus flagellate-induced mortality of picoplankton in three peri-alpine lakes. *J Plankton Res* **31:** 1161–1177.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W.G., Peplies, J., and Glöckner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35:** 7188–7196.

Ram, A.S.P., Nishimura, Y., Tomaru, Y., Nagasaki, K., and Nagata, T. (2010) Seasonal variation in viral-induced mortality of bacterioplankton in the water column of a large mesotrophic lake (Lake Biwa, Japan). *Aquat Microb Ecol* **58:** 249–259.

Roller, C., Wagner, M., Amann, R., Ludwig, W., and Schleifer, K.H. (1994) In-situ probing of gram-positive bacteria with high DNA G+C content using 23S-ribosomal-RNA-targeted oligonucleotides. *Microbiology* **140:** 2849–2858.

Salcher, M.M., Pernthaler, J., and Posch, T. (2010) Spatiotemporal distribution and activity patterns of bacteria from three phylogenetic groups in an oligomesotrophic lake. *Limnol Oceanogr* **55:** 846–856.

Salcher, M.M., Pernthaler, J., Frater, N., and Posch, T. (2011a) Longitudinal and vertical niche separation of different bacterioplankton populations in a large, deep prealpine lake. *Limnol Oceanogr* **56:** 2027–2039.

Salcher, M.M., Pernthaler, J., and Posch, T. (2011b) Seasonal bloom dynamics and ecophysiology of the freshwater sister clade of SAR11 bacteria 'that rule the waves' (LD12). *ISME J* **5:** 1242–1252.

Sekar, R., Pernthaler, A., Pernthaler, J., Warnecke, F., Posch, T., and Amann, R. (2003) An improved protocol for quantification of freshwater *Actinobacteria* by fluorescence *in situ* hybridization. *Appl Environ Microbiol* **69:** 2928–2935.

Shabarova, T., and Pernthaler, J. (2010) Karst pools in subsurface environments: collectors of microbial diversity or temporary residence between habitat types. *Environ Microbiol* **12:** 1061–1074.

Sherr, E.B., Sherr, B.F., and Albright, L.J. (1987) Bacteria – link or sink. *Science* **235:** 88.

Šimek, K., Armengol, J., Comerma, M., Garcia, J.C., Kojecka, P., Nedoma, J., and Hejzlar, J. (2001a) Changes in the epilimnetic bacterial community composition, production, and protist-induced mortality along the longitudinal axis of a highly eutrophic reservoir. *Microb Ecol* **42:** 359–371.

Šimek, K., Pernthaler, J., Weinbauer, M.G., Hornák, K., Dolan, J.R., Nedoma, J., *et al.* (2001b) Changes in bacterial community composition and dynamics and viral mortality rates associated with enhanced flagellate grazing in a mesoeutrophic reservoir. *Appl Environ Microbiol* **67:** 2723–2733.

Šimek, K., Hornák, K., Mašin, M., Christaki, U., Nedoma, J., Weinbauer, M.G., and Dolan, J.R. (2003) Comparing the effects of resource enrichment and grazing on a bacterioplankton community of a meso-eutrophic reservoir. *Aquat Microb Ecol* **31:** 123–135.

Šimek, K., Hornák, K., Jezbera, J., Mašin, M., Nedoma, J., Gasol, J.M., and Schauer, M. (2005) Influence of top-down and bottom-up manipulations on the R-BT065 subcluster of beta-proteobacteria, an abundant group in bacterioplankton of a freshwater reservoir. *Appl Environ Microbiol* **71:** 2381–2390.

Simon, M., and Azam, F. (1989) Protein-content and protein-synthesis rates of planktonic marine-bacteria. *Mar Ecol Prog Ser* **51:** 201–213.

Stamatakis, A., Hoover, P., and Rougemont, J. (2008) A rapid bootstrap algorithm for the RAxML web servers. *Syst Biol* **57:** 758–771.

Tang, K.W., Bickel, S.L., Dziallas, C., and Grossart, H.P. (2009) Microbial activities accompanying decomposition of cladoceran and copepod carcasses under different environmental conditions. *Aquat Microb Ecol* **57:** 89–100.

Tarao, M., Jezbera, J., and Hahn, M.W. (2009) Involvement of cell surface structures in size-independent grazing resistance of freshwater *Actinobacteria*. *Appl Environ Microbiol* **75:** 4720–4726.

Van den Wyngaert, S., Salcher, M.M., Pernthaler, J., Zeder, M., and Posch, T. (2011) Quantitative dominance of seasonally persistent filamentous cyanobacteria (*Planktothrix rubescens*) in the microbial assemblages of a temperate lake. *Limnol Oceanogr* **56:** 97–109.

Warnecke, F., Sommaruga, R., Sekar, R., Hofer, J.S., and Pernthaler, J. (2005) Abundances, identity, and growth state of *Actinobacteria* in mountain lakes of different UV transparency. *Appl Environ Microbiol* **71:** 5551–5559.

Weiss, M., and Simon, M. (1999) Consumption of labile dissolved organic matter by limnetic bacterioplankton: the relative significance of amino acids and carbohydrates. *Aquat Microb Ecol* **17:** 1–12.

Weisse, T., Müller, H., Pintocoelho, R.M., Schweizer, A., Springmann, D., and Baldringer, G. (1990) Response of the microbial loop to the phytoplankton spring bloom in a large prealpine lake. *Limnol Oceanogr* **35:** 781–794.

Xing, P., Hahn, M.W., and Wu, Q.L. (2009) Low taxon richness of bacterioplankton in high-altitude lakes of the eastern tibetan plateau, with a predominance of *Bacteroidetes* and *Synechococcus* spp. *Appl Environ Microbiol* **75:** 7017–7025.

Yarza, P., Richter, M., Peplies, J., Euzeby, J., Amann, R., Schleifer, K.-H., *et al.* (2008) The All-Species Living Tree project: a 16S rRNA-based phylogenetic tree of all sequenced type strains. *Syst Appl Microbiol* **31:** 241–250.

Young, C.C., Lin, S.Y., Arun, A.B., Shen, F.T., Chen, W.M., Rekha, P.D., *et al.* (2009) *Algoriphagus olei* sp. nov., isolated from oil-contaminated soil. *Int J Syst Evol Microbiol* **59:** 2909–2915.

Zeder, M., and Pernthaler, J. (2009) Multispot live-image autofocusing for high-throughput microscopy of fluorescently stained bacteria. *Cytometry A* **75A:** 781–788.

Zeder, M., Peter, S., Shabarova, T., and Pernthaler, J. (2009) A small population of planktonic *Flavobacteria* with disproportionally high growth during the spring phytoplankton bloom in a prealpine lake. *Environ Microbiol* **11:** 2676–2686.

Zeder, M., Kohler, E., and Pernthaler, J. (2010) Automated quality assessment of autonomously acquired microscopic images of fluorescently stained bacteria. *Cytometry A* **77A:** 76–85.

Zubkov, M.V., Tarran, G.A., Mary, I., and Fuchs, B.M. (2008) Differential microbial uptake of dissolved amino acids and amino sugars in surface waters of the Atlantic Ocean. *J Plankton Res* **30:** 211–220.

Zwart, G., Crump, B.C., Agterveld, M., Hagen, F., and Han, S.K. (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. *Aquat Microb Ecol* **28:** 141–155.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Phylogenetic positioning of the 16S rRNA sequences of the family *Cyclobacteriaceae*, sequences marked by an asterisk are not hybridized by the probe Cyc715. Nodes with values < 50% of bootstrap support (100 maximum likelihood trees) were collapsed into multifurcations. The scale bar represents 10% estimated sequence divergence.

Fig. S2. Phylogenetic positioning of the 16 rRNA sequences of the baVI cluster. Asterisks denote sequences that are not targeted by the newly developed probe BaVI-C-663. Nodes with values < 50% of bootstrap support (100 maximum likelihood trees) were collapsed into multifurcations. The scale bar represents 10% estimated sequence divergence.

Table S1. Dates, sampling depth, temperature and concentrations of dissolved PO_4 and NO_3 in the sampled depth during the study.

Table S2. Percentages of the probes HGC69a (Act), BET42a (Beta), CF319a (CF) and Cyc715 (Cyc) to the active community taking up *N*-acetyl-glucosamine (NAG) and Leucine as determined by MAR-FISH. The four periods that were pooled for the data analysis are highlighted with grey and white background colour.

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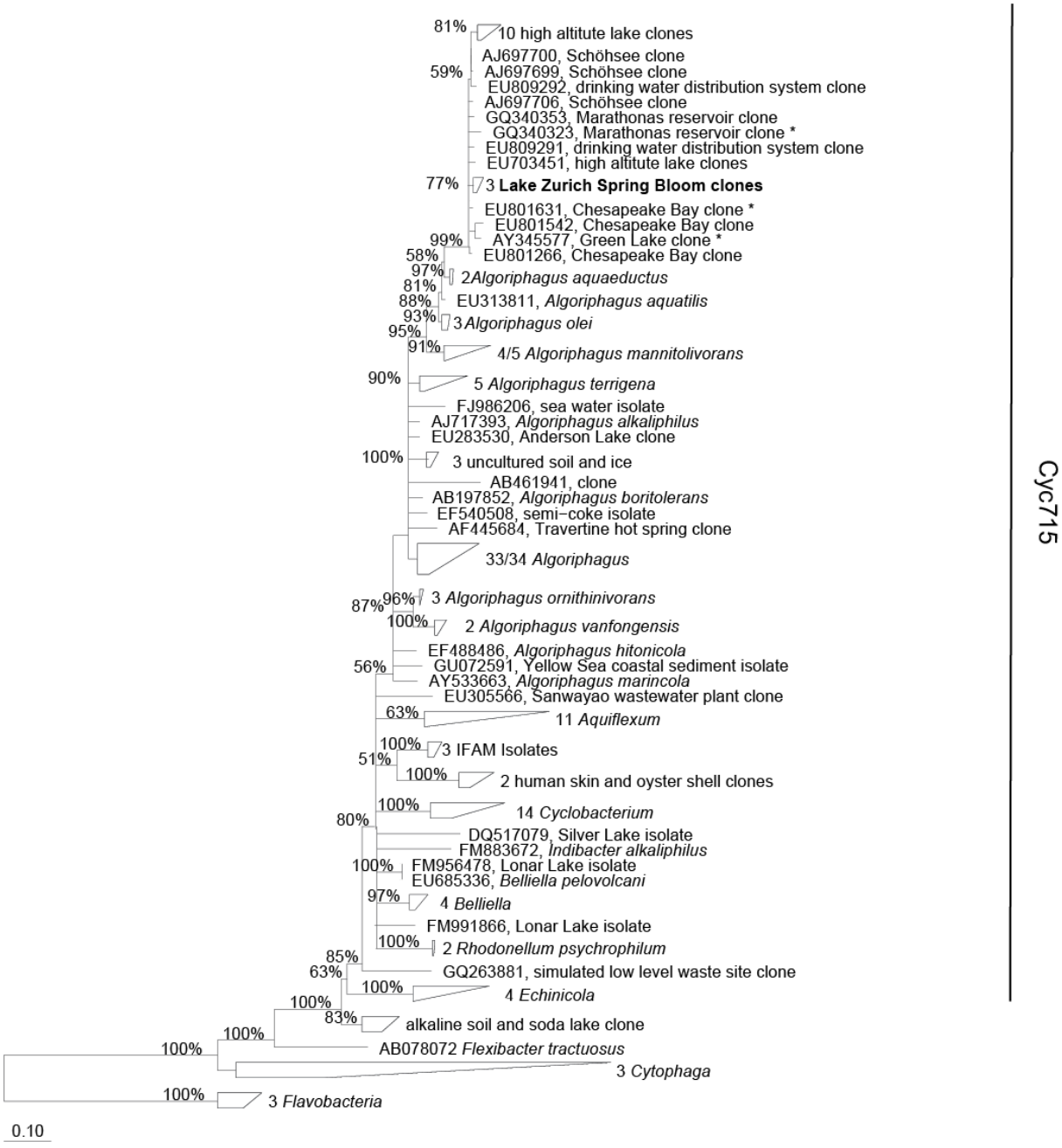

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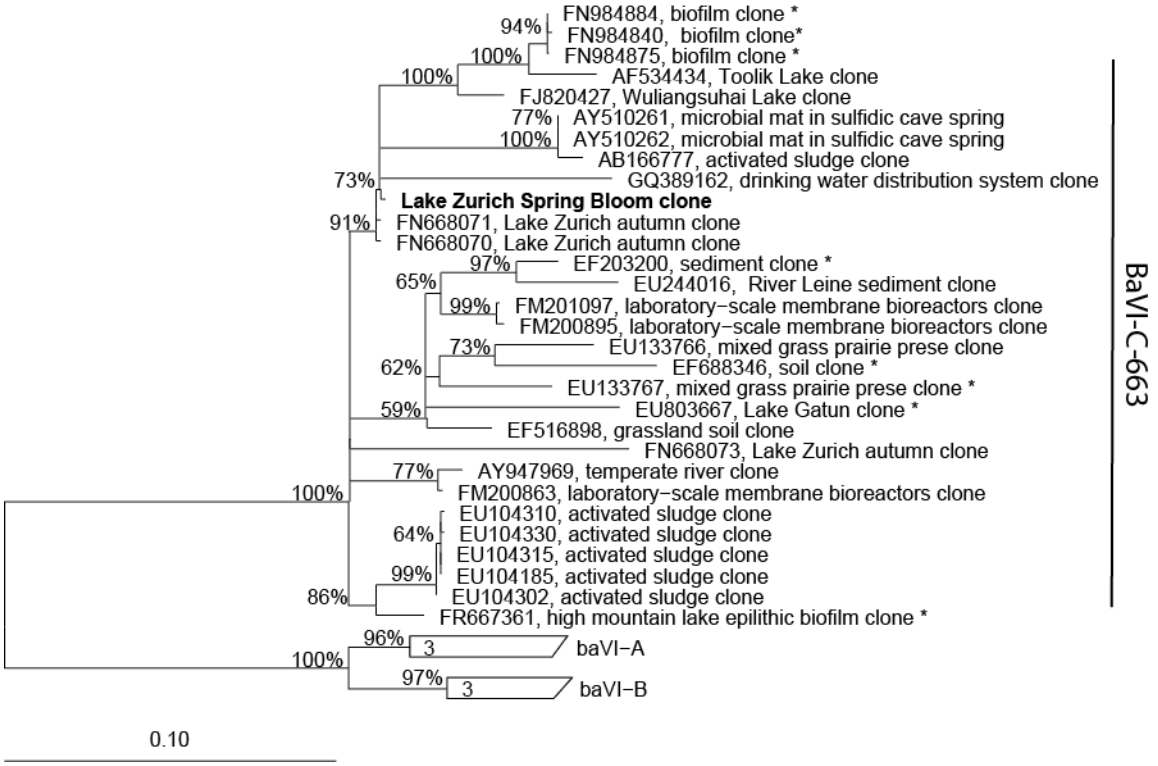

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Table S1. Dates, sampling depth, temperature and concentrations of dissolved PO4 and NO3 in the sampled depth during the study.

Sampling date	**Sampling depth [m]**	**Temperature [°C]**	**PO_4 [µg/l]**	**NO_3 [mg/l]**
13.03.09	6	4	29.7	3.1
18.03.09	5	4.8	25.2	2.8
23.03.09	5.5	4.8	24.5	2.8
30.03.09	4	4.8	29.4	2.9
03.04.09	3.5	4.8	27.4	2.8
07.04.09	3	7.8	9.2	2.6
09.04.09	2	9	3.5	1.8
14.04.09	3	9.8	3.6	2.2
15.04.09	4.5	11	3	2
17.04.09	3	7.7	2.5	2.1
20.04.09	6.5	7.9	1.5	2.4
22.04.09	6.5	9.5	4	1.9
24.04.09	5	10	3.2	1.8
28.04.09	6.5	9	3.4	2.1
30.04.09	10	8.5	2.4	2.2
04.05.09	6.5	8	2	2.2
06.05.09	12	8	4.9	2.2
12.05.09	14	8	4.7	2.4

Tab. S2 Percentages of the probes HGC69a (Act), BET42a (Beta), CF319a (CF) and Cyc715 (Cyc) to the active community taking up N-Acetyl-Glucosamine (NAG) and Leucine as determined by MAR-FISH. The four periods that were pooled for the data analysis are highlighted with grey and white background colour.

Date	% Act		% BETA		%CF		% Cyc	
	NAG	Leucine	NAG	Leucine	NAG	Leucine	NAG	Leucine
03.04.09	4	18	51	60	40	10	5	5
07.04.09	3	15	48	36	41	9	5	6
09.04.09	5	48	57	41	43	7	6	7
14.04.09	41	44	17	30	9	5	2	2
15.04.09	50	38	12	25	9	5	4	4
17.04.09	61	45	17	34	23	10	4	3
20.04.09	6	32	8	58	7	7	11	2
22.04.09	20	66	9	21	3	5	7	3
24.04.09	22	39	17	34	11	6	16	5
28.04.09	4	44	40	34	7	9	18	6
30.04.09	24	49	8	34	1	6	20	7
04.05.09	25	20	12	38	17	9	4	3
06.05.09	61	56	8	35	13	6	18	2
12.05.09	14	30	13	25	26	8	11	2

ARTICLE II

ECKERT EM, BAUMGARTNER M, HUBER IM & PERNTHALER J (2013) GRAZING RESISTANT FRESHWATER BACTERIA PROFIT FROM CHITIN AND CELL-WALL-DERIVED ORGANIC CARBON. ENVIRONMENTAL MICROBIOLOGY 15: 2019-2030.

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Grazing resistant freshwater bacteria profit from chitin and cell-wall-derived organic carbon

Ester M. Eckert, Michael Baumgartner, Iris M. Huber and Jakob Pernthaler*

Limnological Station, Institute of Plant Biology, University of Zurich, Seestrasse 187, 8802 Kilchberg, Switzerland.

Summary

The rise of grazing resistant planktonic bacteria in freshwater lakes during vernal phytoplankton blooms is favoured by predation of heterotrophic nanoflagellates (HNF). The spring period is also characterized by increased availability of organic carbon species that are in parts derived from cellular debris generated during bacterivory or viral lysis, such as peptidoglycan, chitin and their subunit *N*-acetylglucosamine (NAG). We tested the hypothesis that two dominant grazing resistant bacterial taxa, the ac1 tribe of *Actinobacteria* (ac1) and filamentous bacteria from the LD2 lineage (*Saprospiraceae*), profit from such carbon sources during periods of intense HNF predation. The abundances of ac1 and LD2 rose in parallel with HNF, and disproportionally high fractions of cells from both lineages were involved in NAG uptake. Members of ac1 and LD2 were significantly more enriched after NAG addition to lake water. However, highest growth rates of both bacterial lineages were found on chitin and peptidoglycan. Moreover, the direct or indirect transfer of organic carbon from peptidoglycan to LD2 filaments could be demonstrated. We thus provide evidence that these taxa may benefit twofold from protistan predation: by removal of their competitors, and by specific physiological adaptations to utilize carbon sources that are released during grazing or viral lysis.

Introduction

Numerous studies have centred on morphological characteristics and cell wall structure of pelagic freshwater bacteria that have evolved to escape predation by bacterivorous flagellates (e.g. Pernthaler, 2005 and references therein). Inedibility of bacteria has been assigned to cell size and the structure of the cell envelope, suggesting that Gram-positive bacteria, very small cells and long filaments are less efficiently consumed by flagellated predators (Jürgens *et al.*, 1999; Tarao *et al.*, 2009). Consequently, high rates of foraging by heterotrophic nanoflagellates (HNF) may lead to a genotypic succession from medium sized, fast-growing bacteria towards grazing-protected morphologies (e.g. Jürgens *et al.*, 1999; Salcher *et al.*, 2005).

Two phylogenetic lineages dominate the community of grazing resistant bacteria in temperate lakes during spring: The ac1 tribe of *Actinobacteria* (ac1) (Allgaier and Grossart, 2006; Salcher *et al.*, 2010; Eckert *et al.*, 2012) and the LD2 cluster or Aquir tribe affiliated with *Candidatus* Aquirestis calciphila of *Saprospiraceae* (LD2) (Pernthaler *et al.*, 2004; Hahn and Schauer, 2007; Newton *et al.*, 2011). Ac1 is probably the most abundant lineage of grazing protected bacteria in the euphotic zone of temperate lakes, supposedly due to the combined effect of their Gram-positive cell wall and small cell size (Pernthaler *et al.*, 2001; Tarao *et al.*, 2009). These bacteria show parallel temporal dynamics to HNF and especially during later phases of phytoplankton spring blooms constitute the major fraction of the pelagic bacterial community (Weinbauer *et al.*, 2007; Salcher *et al.*, 2010; Eckert *et al.*, 2012). The filamentous LD2 bacteria (Hahn and Schauer, 2007) are numerically much less prominent than ac1, but may considerably contribute to the total prokaryotic biomass due to their large cell size (Pernthaler *et al.*, 2004). These bacteria also show maximal annual abundances during spring when HNF grazing is high and rapidly decline thereafter, presumably due to removal by filter feeding *Daphnia* spp. (Pernthaler *et al.*, 2004; Schauer *et al.*, 2006).

Recent studies suggest an interesting link between ac1 and the amino sugar *N*-Acetylglucosamine (NAG): Beier and Bertilsson (2011) could enrich bacteria affiliated with the ac1 lineage on colloidal chitin, and these ac1 bacteria moreover featured high NAG uptake rates. The composition of the NAG-active community during a phytoplankton bloom underwent a successive shift of dominance with the onset of HNF predation, from fast growing larger bacteria to grazing-resistant ac1 (Eckert *et al.*,

Received 20 September, 2012; revised 12 December, 2012; accepted 3 January, 2013. *For correspondence. E-mail pernthaler@limnol.uzh.ch; Tel. (+41) 44 634 9210; Fax (+41) 44 634 92 25.

2012). A recent genome analysis of a cell from the ac1-B1 subgroup revealed the presence of a gene coding for a chitinase-like protein as well as of other glycoside hydrolases, such as lysozymes, which might also be released extracellularly according to the authors (Garcia *et al.*, 2013).

NAG is the main constituent of chitin, and an important subunit of the bacterial cell wall component peptidoglycan (PG). Chitin is a β-1,4-linked NAG homopolymer; its main lacustrine sources include crustacean carapaces, their faecal pellets, aquatic fungi and centric diatoms (Blackwell *et al.*, 1967; Lee and Fisher, 1992; Tang *et al.*, 2009; Wurzbacher *et al.*, 2010). Chitin seems of particular importance as a substrate in oligotrophic lakes (Köllner *et al.*, 2012). PG is composed of a backbone of alternating β-1,4-linked NAG and N-Acetyl-Muramic acid (NAM) residues which are cross-linked by short-chained oligopeptides. The main sources of PG in aquatic ecosystems are viral lysis, bacterial cell division and HNF grazing (Nagata and Kirchman, 2000; Cloud-Hansen *et al.*, 2006; Middelboe and Jørgensen, 2006). Together with membrane compounds PG is egested by bacterivores as a remnant of incompletely digested prey (Nagata and Kirchman, 1992; Tranvik, 1994), and the amount of cellularly derived DOC may even be altered due to the often co-occuring viral lysis (Middelboe and Jørgensen, 2006; Šimek *et al.*, 2007). It is thus conceivable that the sources of NAG and the composition of DOC and DON in times of high flagellate grazing pressure differs from the previous period (e.g. Andersson *et al.*, 1985; Nagata and Kirchman, 2000; Hasegawa *et al.*, 2001). On the other hand, grazer-mediated shifts in the enzyme activity of the bacterial community (Jürgens and Sala, 2000) seem to indicate that some (e.g. the grazing protected) bacteria may have adapted to exploit these compounds.

Since there are a variety of freshwater bacteria with potentially grazing resistant morphologies (Jürgens *et al.*, 1999; Šimek *et al.*, 2001; Corno and Jürgens, 2008), it is unclear if directly top-down related properties alone can explain the success of members of the ac1 and LD2 lineages during late spring. We thus analysed the uptake of NAG by these grazing resistant bacterial taxa during a phytoplankton spring bloom in Lake Zurich, in order to test for specific adaptations to the substrate conditions that typically arise during periods of high HNF predation. To further explore the notion that these bacteria are specialized on NAG originating from PG, we also used custom radiolabelled crude cell wall extracts (CWEs) from a bacterial culture as a tracer. Additionally we aimed to verify that the LD2 filaments and ac1 *Actinobacteria* in mixed microbial assemblages could disproportionally profit from the addition of NAG and related compounds such as chitobiose (diNAG), chitin, glucose (Glc) and PG.

Results

Abundances

To analyse the dynamics of the abundances of grazing resistant bacteria, we monitored the development of the spring phytoplankton bloom in Lake Zurich and determined the abundances of HNF. Chlorophyll *a* (Chl *a*) concentration rose up to 12.4 mg l^{-1} in the sampled water layer, with a strong decrease towards the end of the study period (3.3 mg l^{-1}). Diatom derived Chl *a* dominated throughout April 2011, representing 48–66% of total Chl *a* concentrations (Fig. 1A). The cyanobacterium *Planktothrix rubescens* constitute another major fraction of the primary producers (1–41% of Chl *a* concentrations). Despite changes in Chl *a* concentration, total bacterial numbers were rather constant (2.5–4×10^6 cells ml^{-1}, Fig. 1B). Stronger fluctuations were observed for HNF numbers, with maximal abundances around 11 April (1.2–8.6×10^3 cells ml^{-1}, Fig. 1B). Members of the ac1 lineage reached abundances of up to 9.5×10^5 cells ml^{-1} shortly thereafter (Fig. 1C). These bacteria constituted a major fraction of the bacterioplankton community, accounting for 19–30% (mean: 25%) of total cells. Filamentous bacterial morphotypes continuously increased in abundance up to 20 April (2.2×10^4 cells ml^{-1}) and were mainly constituted of bacteria from the LD2 tribe (77–92%, mean: 83% of filamentous cells, Fig. 1D). At the onset of May the numbers of LD2 and ac1 decreased to 1.3×10^3 and 6×10^5 cells ml^{-1}, respectively, in parallel to the decrease of Chl *a* concentration and HNF numbers (Fig. 1).

NAG uptake

To evaluate whether high NAG uptake was a more general trait of grazing resistant bacteria, it was determined on five dates with high abundances of ac1 and LD2 (grey shaded area Fig. 1B–D). On average 8 ± 1% of all DAPI stained cells were NAG-active and the total community uptake ranged between 27 and 93 pmol h^{-1} l^{-1}, with highest uptake rates on 27 April (Fig. 2A). The relative contribution of NAG-active ac1 cells was approximately twice as high as the community average (15 ± 2%, Fig. 2B). Highest percentages of active cells were, however, detected within the LD2 tribe: on average 80 ± 7% of all hybridized LD2 cells incorporated the tracer (Fig. 2B).

Enrichments on NAG

Due to the high proportion of LD2 bacteria incorporating NAG in the environment an experiment was conducted to test whether NAG addition would increase their contribution to the bacterial community (Fig. 3). Despite the

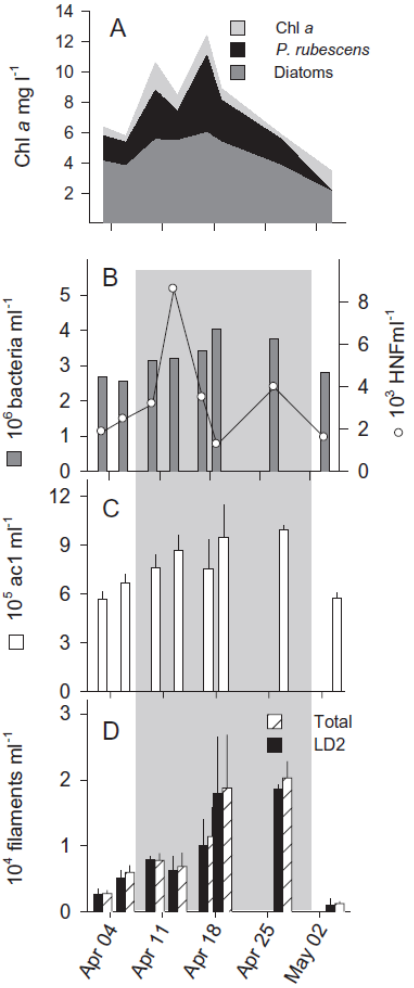

Fig. 1 A. Concentrations of chlorophyll *a* (Chl *a*) of diatoms, of *P. rubescens* and of other phytoplankton in the layer of maximal diatom derived (Chl *a*) in Lake Zurich during spring 2011.
B. Total numbers of HNF and bacteria.
C. Abundances of ac1 *Actinobacteria*.
D. Total numbers of filamentous bacteria and of members of the LD2 tribe of *Saprospiraceae*.
Error bars in this figure and subsequent figures: standard deviations of triplicate samples. The grey shaded area marks the period in which uptake experiments with NAG were conducted.

addition of NAG as a carbon source total bacterial numbers were higher in the control samples (data not shown). The ratio of bacteria per HNF was, however, not significantly different in the two treatments ($P = 0.703$, Fig. 3A), pointing to equal grazing pressure on the bacterial community. LD2 filaments significantly increased both, in relative and absolute abundance in the NAG-amended treatments. By contrast, the population in the control sample did not change compared with the initial time-point, indicating that NAG specifically promoted the growth of LD2 (Fig. 3B and C).

To determine whether LD2 bacteria could also profit from NAG addition in the absence of the competive advantage that they may gain from flagellate grazing, 5000 filamentous bacterial cells were sorted via flow cytometry into media with and without NAG (Fig. 4). Prior to sorting the original assemblage, (maintained on the same NAG containing medium as the sorted cells) consisted of 43% LD2 bacteria and of 25% other filamentous cells. Preliminary analysis suggested that events on cytometric Bi-plots with high DNA fluorescence and high pulse width (a measure that corresponds to the time a cell takes to pass the laser) predominantly corresponded to filamentous morphotypes (data not shown), thus these cells were chosen for sorting (Fig. 4A). The growth of the total community was much more pronounced in the NAG treatment than in the negative control (Fig. 4B), likely because the bacteria were already adapted to NAG as a carbon source. Again, LD2 cells showed a significantly higher abundance in the NAG medium than in the control ($P < 0.001$, Fig. 4B) even in the absence of HNF grazers. LD2 filaments in these enrichments reached abundances of up to 8×10^5 cells ml^{-1}, which is more than one order

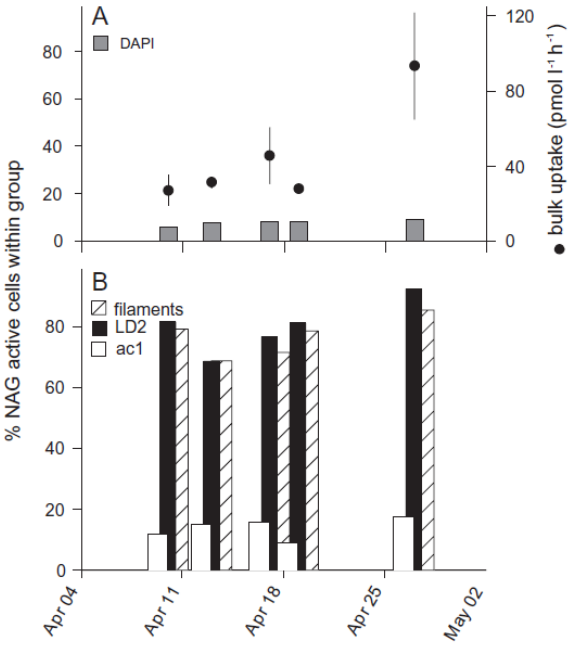

Fig. 2 A. Total community uptake of NAG and percentage of NAG incorporating cells within all DAPI stained cells (DAPI).
B. Proportions of NAG incorporating filamentous bacteria (filaments), of members of the LD2 tribe (LD2) and of ac1 *Actinobacteria* (ac1).

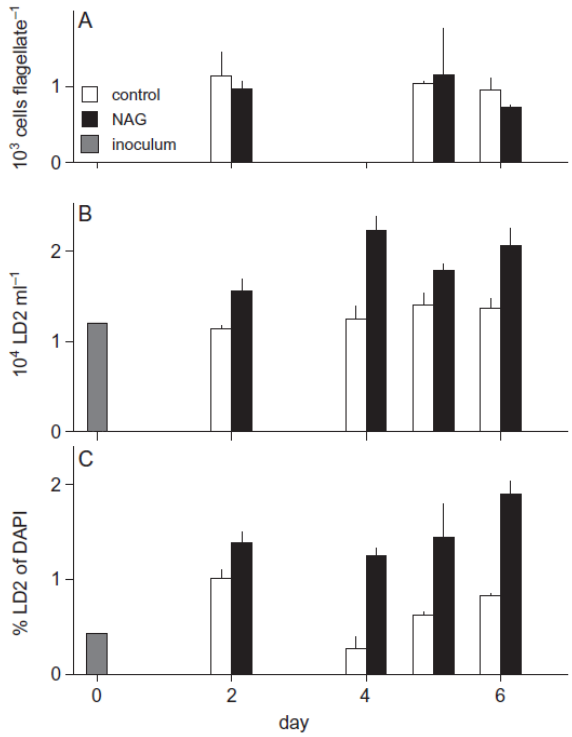

Fig. 3. Enrichment of LD2 bacteria on NAG.
A. Ratio of total bacterial to HNF abundances.
B. Total numbers and (C) relative abundances of LD2 filaments in 5 µm prefiltered lake water enrichments with and without addition of NAG. Difference between treatments: $P < 0.001$.

of magnitude higher then usually found in Lake Zurich (Figs 4B and 1).

Uptake of NAG-related compounds

To gain direct evidence for the transfer of organic carbon from cell wall material (presumably PG) to LD2, a ^{3}H-NAG-labelled bacterial CWE was used as a radiotracer (Fig. 5A). Community uptake rate of CWE was very low (1.3 pmol h^{-1} l^{-1} after 120 h, Fig. 5B). Nevertheless, radiolabelled LD2 bacteria were already detected after 1 day of incubation and their proportion rose to 30% after 5 days (Fig. 5A and B).

We additionally tested if LD2 and ac1, besides the monomer NAG, would incorporate the dimer diNAG and the NAG-precursor Glc. Similar proportions of cells were found to incorporate NAG as observed previously (Fig. 2), with 74% and 15% active LD2 and ac1 cells respectively (Fig. 5C). The percentage of cells with visible incorporation of Glc within the LD2 and ac1 lineages was even higher (84 and 45%). DiNAG was incoporated by 38% of LD2 filaments and 10% of ac1 *Actinobacteria*. The proportion of active cells within the two bacterial groups was above the community average for all substrates (Fig. 5C).

Enrichments on related compounds

Another enrichment experiment was designed to test if the high activity of grazing resistant bacteria was limited to NAG or if their growth could also be enhanced by related molecules such as Glc, and by NAG-containing di- and polymers, i.e. diNAG, chitin and PG (Fig. 6). The overall development of the bacterial community in all treatments was highly different (Fig. 6A). Surprisingly, Glc hardly promoted bacterial growth and total abundances were similar to those observed in the diNAG treatment and the negative control (between 3.3×10^5 and 1.1×10^6 cells ml^{-1}). Cell numbers in the NAG treatment were approximately twice as high (9.5×10^5–2.6×10^6 cells ml^{-1}). In marked contrast, the two polymers PG and chitin lead to very high cell numbers, with maxima of 2.7×10^7 and 5×10^6 cells ml^{-1}, respectively, on day 6. Consequently

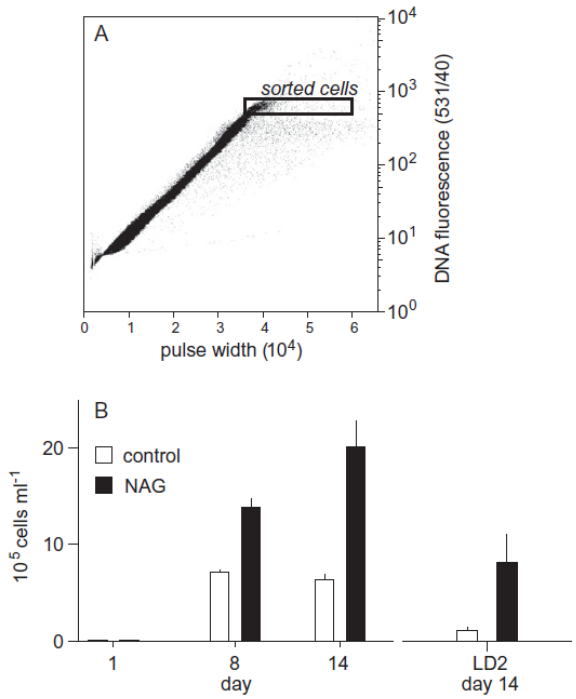

Fig. 4. Enrichment of LD2 bacteria after flow cytometric separation from HNF.
A. Scatter bi-plot of DNA fluorescence (531 nm) vs pulse width. Cells selected for subsequent sorting are marked with a black frame.
B. Total cell numbers sorted into media with and without NAG, and numbers of LD2 filaments after 2 weeks of incubation. Note that the initially sorted numbers of cells (5000) were too low to appear in the depiction (day 1).

HNF predation pressure also differed in the treatments (Fig. 6A). While the negative control, Glc-, NAG- and diNAG treatment had similar ratios of bacteria to HNF, this proportion was lower in the peptidoglycan treatment throughout the first 6 days. By contrast, very high numbers of HNF were detected in the chitin samples already on day 2.

Abundances of LD2 in the NAG treatment significantly exceeded those of the negative control ($P = 0.005$, Fig. 6B). NAG rapidly promoted the growth of LD2: an eightfold increase in filament numbers (to 8.2×10^3 cells ml^{-1}) was already detected after 2 days of incubation. By contrast, a significant increase of LD2 in the negative control was only found on day 11 (7.2×10^3 cells ml^{-1}, Fig. 6B). Despite the high percentages of Glc-active LD2 cells in the preceding tracer experiment (Fig. 5C), this substrate did not significantly promote their growth in the experimental community ($P = 0.151$, Fig. 6B). Growth of LD2 filaments on diNAG was somewhat slower then on NAG: their abundances only doubled within the first 2 days, and a pronounced increase was only detected after 4 days. The addition of chitin promoted continuous growth of LD2 bacteria, and the highest relative and absolute abundances of LD2 ever observed were detected in this treatment on day 11 (6.6×10^4 cells ml^{-1}, corresponding to 2% of total cells). When grown on PG LD2 multiplied in abundance by a factor of 24 within 2 days. Unlike the chitin treatments, the increasing population in PG-amended incubations was not stable, and a decrease of LD2 cells was observed after day 4 (from 3.6×10^4 cells ml^{-1} on day 4 to 0.6×10^4 cells ml^{-1} on day 11).

NAG significantly promoted ac1 growth over the first 4 days (to 2.6 and 2.4×10^5 cells ml^{-1} on days 2 and 4 respectively), whereas their population size decreased thereafter (0.8×10^5 cells ml^{-1} on day 11). None of the provided substrates stimulated continuous growth of ac1 over the course of the experiment, and their relative contribution to the bacterial community decreased in all treatments (Fig. 6C). As observed for LD2, ac1 development in the Glc treatment did not significantly differ from the negative controls ($P = 0.689$, Fig. 6C). The abundances of ac1 in diNAG treatments were akin to the ones observed in the negative control ($P = 0.765$, Fig. 6C). Chitin, however, strongly promoted ac1 growth and lead to a population of 5–7.2×10^5 cells ml^{-1} over the course of the first 6 days. Reduced cell numbers were only observed on day 11 (2×10^5 cells ml^{-1}). As observed for LD2 filaments, the highest increase of ac1 abundances was observed in the PG treatment during the first 2 days (22-fold, to 2.2×10^6 cells ml^{-1}). However, ac1 numbers declined already on day 4, and this was even more pronounced during the subsequent days (> 90% reduction on day 6 compared with day 2).

Discussion

Amino sugars and pelagic bacteria

During spring phytoplankton blooms, periods of rapid growth of the microbial assemblages alternate with periods of high mortality rates (Weisse *et al.*, 1990). Bacterial division (Cloud-Hansen *et al.*, 2006) and mortality, due to viral lysis or flagellate grazing, causes the release of NAG and PG (Nagata and Kirchman, 2000; Riemann and Middelboe, 2002; Kawasaki and Benner, 2006). In addition, the growth and lysis of centric diatoms may release NAG and chitin (Blackwell *et al.*, 1967). Thus an adaptation to these amino sugars might serve as a clear advantage for heterotrophic bacteria during spring time.

Although only a low fraction of aquatic bacteria is capable of chitin hydrolysis, its turnover in freshwater habitats is nevertheless high (Beier *et al.*, 2012; Köllner *et al.*, 2012). The addition of chitin to lake water samples had a pronounced impact on bacterial growth (Fig. 6), indicating that the chitinolytic community was limited by the *in situ* availability of this substrate (Kang *et al.*, 2005; Beier *et al.*, 2012).

Substantial enrichment of a natural bacterial assemblage on PG has previously been observed in an estuarine environment (Jørgensen *et al.*, 2003), but not in freshwaters (Fig. 6). It is important to emphasize that the PG used in our enrichment experiments did not only consist of the NAG–NAM backbone, but also contained the amino acid residues. Since the peptide compounds are likely degraded more rapidly than the polysaccharide moiety, the amino acid chains may have additionally boosted the growth of the experimental bacterial assemblage (Nagata *et al.*, 2003). Nevertheless, it seems that some bacteria are also capable of recycling the PG backbone (Nagata *et al.*, 2003; Kawasaki and Benner, 2006). Apart from the description of two PG degrading freshwater isolates (Jørgensen *et al.*, 2009; 2010), the majority of studies about PG degradation have centred on its *in situ* turnover rates of the molecule (e.g. Jørgensen *et al.*, 2003; Nagata *et al.*, 2003), and very little is known about the identity of bacteria involved in this process. In this study we present evidence for the involvement of two important grazing resistant bacterial taxa in the turnover of both chitin and PG in freshwater ecosystems.

LD2 filaments

NAG uptake is considered widespread among microbial phyla; however, only a small percentage of the natural bacterial community are usually NAG-active and total uptake is low compared with substrates such as amino acids (Nedoma *et al.*, 1994; Beier and Bertilsson, 2011; Eckert *et al.*, 2012). Since the concentrations of the sub-

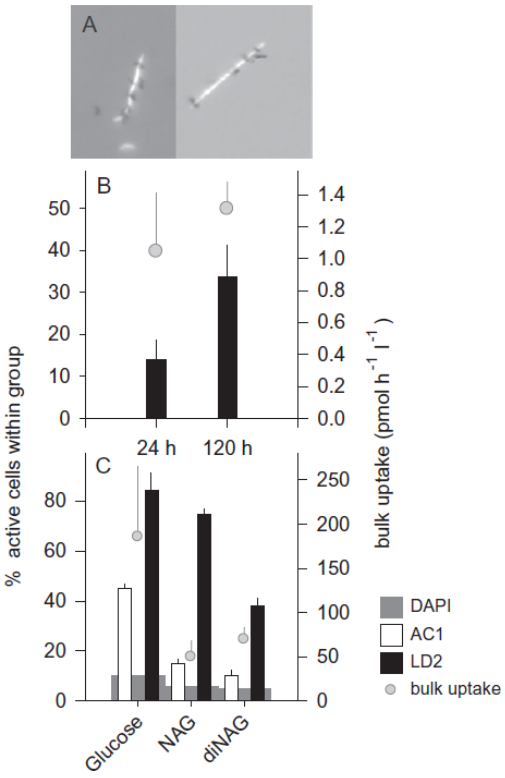

Fig. 5 A. Photomicrographs of MAR-FISH preparations of LD2 which incorporated radiolabelled cell wall extract.
B. Total community uptake of cell wall extract and percentage of active cells within the LD2 tribe.
C. Total community uptake of NAG, diNAG and glucose and percentage of active cells within all DAPI stained cells, the LD2 and ac1 tribe.

strates in lake water were not determined, it is not possible to assess the potentially negative effect of isotope dilution on the detection sensitivity of tracer uptake (King and Berman, 1984), which might be a reason for the seemingly low NAG uptake. However, we show that a vast majority of LD2 were actively incorporating NAG as well as Glc and a substantial fraction also assimilated diNAG (Figs 2 and 5). LD2 bacteria and freshwater *Actinobacteria* contributed to a similar extend to total prokaryotic biomass in Lake Zurich surface waters towards the end of April [assuming cell sizes of approximately 1 μm^3 and 0.025 μm^3 respecively (Pernthaler *et al.*, 2004; Posch *et al.*, 2009)]. This illustrates that despite their low abundances LD2 filaments may be considerably involved in carbon turnover. Moreover, amino sugars significantly promoted growth of these bacteria in mixed assemblages with and without flagellate grazers and independently of the growth behaviour of the total community (Figs 3, 4 and 6). Highest growth rates of LD2 were detected when amino sugar containing polymers were added as substrates (Fig. 6). Since these bacteria are known as pelagic, free-living cells (Hahn and Schauer, 2007), their apparent affinity to polymers is somewhat counterintuitive. A phylogenetically closely related lineage to LD2 are affiliated with the cultured filamentous bacterium *Haliscomenobacter hydrossis* (HAL-A1 tribe; Newton *et al.*, 2011), commonly found in waste water treatment plants (van Veen *et al.*, 1973). Interestingly, also HAL-A1 bacteria were highly NAG-active and, albeit pelagic, were observed to have chitinase activity (Kämpfer, 1995; Kragelund *et al.*, 2008). It is thus possible that LD2 are likewise capable of chitin hydrolysis. Our study also provides evidence for a transfer of organic carbon from bacterial cell walls to LD2 bacteria (Fig. 5). This raises the question whether LD2 directly lysed peptidoglycan or chitin, or if the uptake was related to polymer degradation and release of monomers by sympatric bacteria, as has been described in other studies (reviewed by Velicer, 2003). The backbone of chitin and PG are of similar chemical nature and many chitinases and lysozymes are known to hydrolyse both, the PG backbone and chitin (e.g. Wang and Chang, 1997). Thus a potential chitinolytic activity of LD2 could also imply the ability to lyse PG.

The growth pattern of LD2 over time in the chitin and PG treatment were markedly different (Fig. 6). The filaments appeared to profit more rapidly from PG, but the population was also strongly diminished in the later phase of the experiment. Mortality of LD2 is usually assigned to *Daphnia* spp. filter feeding (Pernthaler *et al.*, 2004; Schauer *et al.*, 2006). However, zooplankton-induced mortality in the incubation experiments can be excluded due to the 5 µm pre-filtration step. Thus it is unclear which factor reduced the LD2 population in the PG enrichments. It is, e.g. conceivable that LD2 mortality was related to the presence of small unicellular eukaryotes other than flagellates, such as ciliates and amoeba, which could not be observed in any other treatment (E. Eckert, pers. observation).

ac1 Actinobacteria

Although there are an increasing number of studies that report amino sugars as a substrate for ac1 *Actinobacteria*, their specific role in the turnover of such compounds remains under debate (Beier and Bertilsson, 2011; Eckert *et al.*, 2012; Garcia *et al.*, 2013). For example, while it has been hypothesized that ac1 *Actinobacteria* are able to take up NAG but do not hydrolyse chitin, a chitinase-like gene has been found in an ac1 genome (Beier and Bertilsson, 2011; Garcia *et al.*, 2013). Here we provide additional experimental evidence for the involement of ac1 in chitin degradation and we additionally propose their involvement in PG turnover.

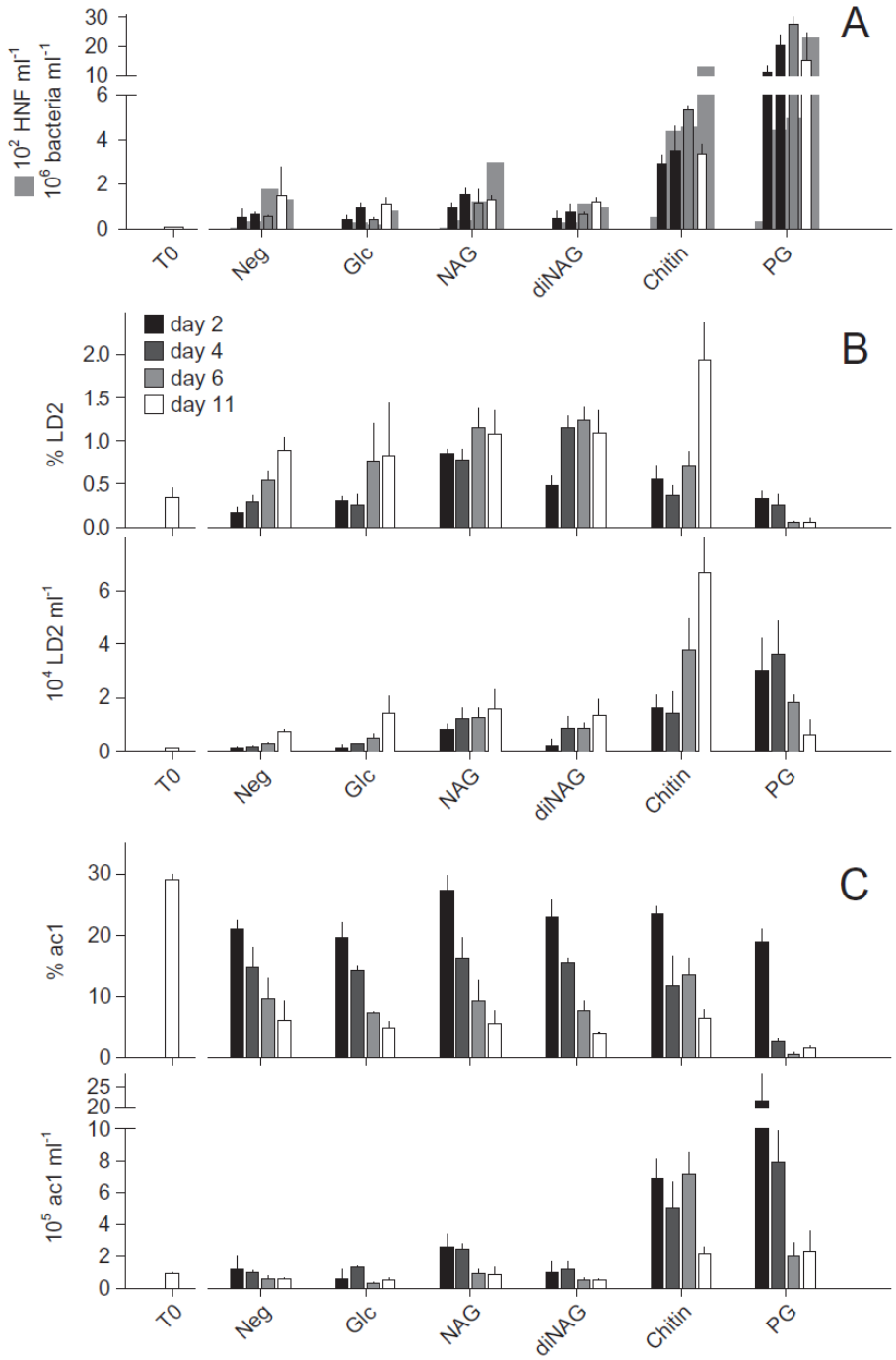

Fig. 6. Enrichment of LD2 and ac1 on different mono- and polymers. (A) Absolute bacterial and heterotrophic flagellate numbers; (B) proportions (upper panel) and absolute abundances (lower panel) of LD2 filaments and (C) proportions (upper panel) and absolute abundances (lower panel) of ac1 *Actinobacteria* in 5 µm prefiltered lake water enrichments with no added substrate (Neg), and after addition of Glc, NAG, diNAG, chitin and PG.

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While the disproportionally high NAG activity of ac1 *Actinobacteria in situ* (compared with the community average) (Fig. 2) is in line with previous findings (Beier and Bertilsson, 2011; Eckert *et al.*, 2012). However, only a transient enrichment of these bacteria could be achieved after addition of NAG (Fig. 6), which may hamper their eventual isolation using this substrate. As already described by Beier and Bertilsson (2011), the uptake of diNAG was rather low, and this substrate did not promote their abundances in our enrichment experiments (Figs 5 and 6). By contrast, growth of ac1 bacteria was strongly stimulated by the two polymers (Fig. 6). A recent genomic analysis of an ac1-cell identified a chitinase-like protein as well as many lysozymes (Garcia *et al.*, 2013). If these findings are set in the context of our experimental observation of higher ac1 growth on chitin and PG than on NAG, there is strong overall evidence that these bacteria might indeed act as primary degraders of the polymers (Fig. 6). Based on their comparably low diNAG uptake (Fig. 5C), it has been hypothesized that ac1 do not synthetize chitinases themselves, but profit from the NAG release by exoenzymatic activity of others (Beier and Bertilsson, 2011). Since many chitinases release dimers when lysing the polymer, the ability of a bacterium to take up diNAG has been used as an indicator for its production of this enzyme. However, not all bacterial chitinases release diNAG (e.g. Cohen-Kupiec and Chet, 1998). Thus, bacteria may not express pathways for diNAG acquisition if they possess chitinases that free monomers or polymers greater than two subunits. This notion is further supported by the observation that the addition of diNAG only weakly stimulated bacterial growth compared with chitin (Fig. 6). Hence, it is unlikely that all chitinolytic bacteria were able to acquire diNAG, which might also apply to members of the ac1 lineage. Nevertheless, Beier and Bertilsson (2011) have convincingly demonstrated that ac1 bacteria do not attach to chitin particles. This finding is further confirmed by a phylogenetic study from a lake showing that particle associated *Actinobacteria* usually differ from the free-living fraction (Rösel and Grossart, 2012). Assuming that many size classes of amino sugar containing polymers are released during HNF feeding, the polymeric substrates available to *Actinobacteria* may be of enzymatically predigested nature (Vrba *et al.*, 1993). In fact, PG has previously been observed to be digested into small units before it is egested by flagellates (Tranvik, 1994). Thus, the ability for attachment may not be required for PG degradation by free-living ac1 bacteria. Unfortunately, uptake of radiolabelled CWE by ac1 could not be determined for technical reasons: Due to the relatively small amount of incorporated label, long development times of the photographic emulsion were required to obtain clear signals. This resulted in a higher background and in too many potential false-positive signals associated with the small ac1 cells. It might be possible to eventually overcome this problem by using ^{14}C- instead of ^{3}H-labelled NAG, in order to obtain a stronger labelling of the CWE.

Regardless of the substrate, ac1 lost remarkably in importance towards the end of all the incubations (Fig. 6). To date, the main cause of mortality of this actinobacterial population remains unknown. It has been speculated that viral lysis could play an important role (Weinbauer *et al.*, 2007), which can neither be confirmed nor excluded by our experimental set-up.

Conclusions

Although it remains to be explored whether ac1 or LD2 act as primary polymer degraders, our study clearly indicates that these pelagic bacteria are closely linked to polymer degradation and that they can profit from PG, NAG and chitin released into the pelagic zone. It thus appears that these grazing resistant taxa have developed specific physiological adaptations to sequester carbon substrates released by the lysis of those bacterial populations that are more vulnerable to viruses and/or protistan predators.

Experimental procedures

Sampling

Lake Zurich an oligo-mesotrophic subalpine lake was sampled twice a week between 3 April and 4 May 2011 at approximately 10:00 h and coordinates 47°31′ N, 8°58′ E. Chl *a* was determined directly on the boat using a multiple wavelength fluoroprobe (TS-16–12 fluoroprobe, bbeMoldaenke GmbH, Kronshagen, Germany) which was calibrated to distinguish fluorescence at six wavelengths (370, 470, 525, 570, 590, 610 nm) in order to assign these fluorescence fingerprints to different algal taxa. Two litres of sample were taken from the layer of maximum diatom derived Chl *a* (between 3.5 and 8 m depth) and transported to the laboratory in a cooling box within 40 min.

Samples for fluorescence *in situ* hybridization and catalysed reporter deposition (CARD-FISH) and for flow cytometric analysis were fixed with a buffered paraformaldehyde solution (PFA, 1% final concentration). Flow cytometry samples were stored at 4°C until further analysis. Three times 5 ml of sample were fixed for 1 h at room temperature, filtered on 0.2 µm filters (GTTP, 45 mm diameter, Millipore) and stored at −20°C until used for CARD-FISH analysis. For HNF counting 40 ml of lake water were fixed with alkaline Lugol's solution (0.5% final concentration), formaldehyd (FA, 3% final concentration), decolourized with several drops of sodium thiosulfate and stored at 4°C until filtration and counting (Sherr and Sherr, 1993).

Bacterial and HNF abundances

Total bacterial abundances were determined by flow cytometry (inFlux V-GS, Becton Dickinson, Franklin Lakes, NJ,

USA). One millilitre of FA-fixed samples was stained with 10 µl of 4′,6-Diamidino-2-phenylindole (DAPI, 1 µg ml^{-1} final concentration). A UV laser (60 mW, 355 nm) served as excitation source and DAPI emission was measured at 460 ± 50 nm. SSC was determined with a blue laser (488 nm). Scatter bi-plots of DNA fluorescence and SSC were analysed using an in-house software (J. Villiger, unpublished).

Relative abundances of LD2 and ac1 bacteria were determined by CARD-FISH analysis as described by Sekar and colleagues (2003). Since the LD2 filaments were highly prone to over digestion fixation of the enrichment samples was prolonged to 2 h at RT, and the digestion step for CARD-FISH of LD2 was reduced to 20 min of lysozyme treatment. The oligonucleotide probes used were: LD2-739 for bacteria affiliated with *Candidatus* Aquirestis calciphila (Pernthaler *et al*., 2004) and ac1-852 for the ac1 lineage of *Actinobacteria* (Warnecke *et al*., 2005). Total numbers of filamentous bacteria and of members of the LD2 subcluster were counted manually (minimum 10 fields and 1000 bacterial cells). Bacteria hybridized with ac1-852 were counted using an automated microscopic platform (AxioImager Z1) (Zeder and Pernthaler, 2009).

For the counting of HNF 10–40 ml of subsamples were stained with DAPI for 7 min and filtered onto black 1 µm pore size polycarbonate filters (25 mm diameter, GE Water & Process Technologies Trevose, PA, USA). At least 50 HNF cells or 100 fields were counted manually per sample at 400× magnification.

Substrate incorporation

Community bulk incorporation and uptake by specific phylogenetic groups of Glc, NAG and diNAG was evaluated using tritium-labelled substrates (for CWE see below). Fifty millilitres of lake water was filtered through a 30 µm plankton net to remove zooplankton grazers. Triplicate 10 ml samples as well as 5 ml pre-killed control were incubated at *in situ* temperature with 10 nM D-[5,6-^{3}H]-Glc (specific activity 60 Ci mmol^{-1}), [1,6-^{3}H(N)]-NAG (specific activity; 60 Ci mmol^{-1}) or [^{3}H(G)-N,N]-diNAG (specific activity: 5 Ci mmol^{-1}, American Radiolabelled Chemicals, St Louis, Mo, USA) for 3 h before fixation with buffered PFA (2% end concentration).

Microautoradiography combined with CARD-FISH (MAR-FISH) was conducted as described in Alonso and Pernthaler (2005) with modification according to Eckert and colleagues (2012). Bulk community uptake was determined as described by Kirchman (2001) using the ice-cold trichloroacetic acid extraction method. The incorporated radioactivity was quantified on a scintillation counter (Tri-Carb 3170TR/SL, PerkinElmer, Waltham, MA, USA).

Incubations with radiolabelled cell wall extract

Since radiolabelled CWE was not commercially available we extracted it from a culture grown on radiolabelled NAG. A *Flavobacteria* sp. culture (Accession No.: HE802578) was grown for 20 h (beginning of stationary growth phase) in 10 ml medium containing 0.5 g l^{-1} Casein hydrolysate (Fluka analytics), the inorganic components of the R2A Medium (Reasoner and Geldreich, 1985) and 100 µCi of ^{3}H-NAG. Casein hydrolysate was chosen following the suggestion of Hancock and Poxton (1988) to avoid additional input of amino sugars and ensure an incorporation of the radiolabelled NAG into the cell wall. Crude cell walls were subsequently extracted as described by Nagata and colleagues (2003). In brief; cells were pelleted and washed three times with sterile 0.5× PBS before being lysed in 35 ml 2.5% SDS buffer in a 98°C water bath for 1 h. The lysate was subsequently centrifuged in an ultracentrifuge (Sorvall RC 50 plus, Thermo Scientific, Waltham, MA, USA) at 49 500 *g* for 45 min in 50 ml polycarbonate vials (OAK Ridge PC, 50 ml, Nalgene, Rochester, NY, USA). The crude CWE was washed three times with sterile MQ and stored in 500 µl PCR grade water at −20°C.

The approximate amount of amino sugar in the extracted cell walls was determined from a replicate culture with unlabelled NAG by a colorimetric method in a 96-well plate (Cheng *et al*., 2011). Calibrations were made using NAG in different concentrations and different amounts of chitin. The amount of amino sugars in the cell wall extracted from 100 ml of culture corresponded to approximately 1 mg of chitin. Radioactivity of the substrate was determined using triplicate 100 µl of the dissolved CWE in a scintillation counter, and was around 46 nM radiolabelled NAG in 100 µl CWE.

On 27 April 2011, nine samples (plus three pre-killed controls, 4.75 ml each) of lake water (prefiltered by 30 µm) were incubated with 20 µl of labelled CWE at *in situ* temperature. Triplicate incubations and one negative control were fixed with FA (2% final concentration) after 3, 24 and 120 h respectively. 1.75 ml per sample was used for MAR-FISH and 3 ml for the determination of bulk community incorporation as described above.

Enrichment of LD2 on NAG

On 12 April 2011 an experiment was performed to determine whether LD2 bacteria would increase in abundance with the addition of NAG. For this purpose 600 ml of lake water were filtered through a 5 µm pore size polycarbonate filter (47 mm diameter, GE Water & Process Technologies Trevose, PA, USA) to remove larger grazers and in turn stimulate HNF growth (Šimek *et al*., 2001). Triplicate subsamples of 100 ml were incubated either after addition of NAG (250 µM, Sigma-Aldrich) or without substrate addition (negative control) in 300 ml Erlenmeyer flasks. All flasks were kept at 14°C in the dark without shaking and sampled every 1–2 days. Total abundances of bacteria, the contribution of LD2 and the number of flagellates were determined as described above.

Enrichment of LD2 without flagellates (flow cytometric cell sorting)

To investigate whether LD2 could also be enriched without flagellates, we chose to sort filamentous morphologies with a flow cytometer and determine their growth with and without NAG addition. For this purpose 1 ml of unfixed sample from an enrichment of filamentous LD2 bacteria was stained with 10 µl of SYBR GREEN (1 µg ml^{-1} final concentration, Invitrogen, Oregon, USA) and sorted using flow cytometry. A blue laser served as extinction source (488 nm). Cells were sorted by comparing scatter Bi-plots of DNA fluorescence and SSC

pulse width. We reckoned that filamentous bacteria had a higher pulse width signal compared with rod shape ones due to their longer passage time though the point of interrogation; thus such cells were chosen for sorting (Fig. 4). Six times 5000 cells were sorted into sterile 2 ml tubes and immediately diluted into 1 ml of medium before being further transferred to 10 ml of medium in 50 ml culture flasks (Nunclon Δ Surface, Nunc, Roskilde, Denmark). The medium was composed of unfiltered pre-autoclaved lake water amended with 0.4 mg l^{-1} thiamine and 0.1 mg l^{-1} vitamin B12 (Calbiochem, Darmstadt, Germany) to triplicate control samples, and additionally with 250 μM of NAG for triplicate NAG-treated samples. Cells were grown at 18°C with shaking and analysed for growth after 8 and 14 days using flow cytometry and CARD-FISH as described above.

Enrichment of LD2 and ac1 on different mono- and polymeres

We also tested whether LD2 and ac1 could be enriched on substrates related to NAG; diNAG, PG (from *Bacillus subtilis*), chitin (all Sigma-Aldrich, St Louis, MO, USA) as well as on Glc (Merck KGaA, Darmstadt,Germany). On 12 April 2012 lake water from 5 m depth was filtered through 5 μm pore size filters as described above. For each substrate triplicate 40 ml samples were incubated at 14°C in the dark and sampled after 2, 4, 6 and 11 days. Triplicate 40 ml samples without substrate addition served as controls. Before the incubations incorporation of radiolabelled Glc, NAG and diNAG was assessed as described above. Total bacterial numbers were counted by flow cytometry as described above except for PG samples that were evaluated microscopically after DAPI staining due to the formation of flocks that would clog the flow cytometer capillary tubes. Populations of HNF, LD2 and ac1 were counted as described above.

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References

Allgaier, M., and Grossart, H.P. (2006) Diversity and seasonal dynamics of Actinobacteria populations in four lakes in northeastern Germany. *Appl Environ Microbiol* **72:** 3489–3497.

Alonso, C., and Pernthaler, J. (2005) Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. *Appl Environ Microbiol* **71:** 1709–1716.

Andersson, A., Lee, C., Azam, F., and Hagstrom, A. (1985) Release of amino-acids and inorganic nutrients by heterotrophic marine microflagellates. *Mar Ecol Prog Ser* **23:** 99–106.

Beier, S., and Bertilsson, S. (2011) Uncoupling of chitinase activity and uptake of hydrolysis products in freshwater bacterioplankton. *Limnol Oceanogr* **56:** 1179–1188.

Beier, S., Mohit, V., Ettema, T.J.G., Östman, Ö., Tranvik, L.J., and Bertilsson, S. (2012) Pronounced seasonal dynamics of freshwater chitinase genes and chitin processing. *Environ Microbiol* **14:** 2467–2479.

Blackwell, J., Parker, K.D., and Rudall, K.M. (1967) Chitin fibers of diatoms *Thalassiosira fluviatilis* and *Cyclotella cryptica*. *J Mol Biol* **28:** 383–385.

Cheng, Y.S., Labavitch, J., and VanderGheynst, J.S. (2011) High-throughput analysis of hexosamine using a colorimetric method. *Anal Biochem* **408:** 160–162.

Cloud-Hansen, K.A., Peterson, S.B., Stabb, E.V., Goldman, W.E., McFall-Ngai, M.J., and Handelsman, J. (2006) Breaching the great wall: peptidoglycan and microbial interactions. *Nat Rev Microbiol* **4:** 710–716.

Cohen-Kupiec, R., and Chet, I. (1998) The molecular biology of chitin digestion. *Curr Opin Biotechnol* **9:** 270–277.

Corno, G., and Jürgens, K. (2008) Structural and functional patterns of bacterial communities in response to protist predation along an experimental productivity gradient. *Environ Microbiol* **10:** 2857–2871.

Eckert, E.M., Salcher, M.M., Posch, T., Eugster, B., and Pernthaler, J. (2012) Rapid successions affect microbial N-acetyl-glucosamine uptake patterns during a lacustrine spring phytoplankton bloom. *Environ Microbiol* **14:** 794–806.

Garcia, S.L., McMahon, K.D., Martinez-Garcia, M., Srivastava, A., Sczyrba, A., Stepanauskas, R., *et al*. (2013) Metabolic potential of a single cell belonging to one of the most abundant lineages in freshwater bacterioplankton. *ISME J* **7:** 137–147.

Hahn, M.W., and Schauer, M. (2007) '*Candidatus* Aquirestis calciphila' and '*Candidatus* Haliscomenobacter calcifugiens', filamentous, planktonic bacteria inhabiting natural lakes. *Int J Syst Evol Microbiol* **57:** 936–940.

Hancock, I.C., and Poxton, I.R. (eds) (1988) *Bacterial Cell Surface Techniques*. Chichester, UK: Wiley.

Hasegawa, T., Koike, I., and Mukai, H. (2001) Release of dissolved organic nitrogen by a planktonic community in Akkeshi Bay. *Aquat Microb Ecol* **24:** 99–107.

Jørgensen, N.O.G., Stepanaukas, R., Pedersen, A.G.U., Hansen, M., and Nybroe, O. (2003) Occurrence and degradation of peptidoglycan in aquatic environments. *FEMS Microbiol Ecol* **46:** 269–280.

Jørgensen, N.O.G., Brandt, K.K., Nybroe, O., and Hansen, M. (2009) Delftia lacustris sp nov., a peptidoglycan-degrading bacterium from fresh water, and emended description of *Delftia tsuruhatensis* as a peptidoglycan-degrading bacterium. *Int J Syst Evol Microbiol* **59:** 2195–2199.

Jørgensen, N.O.G., Brandt, K.K., Nybroe, O., and Hansen, M. (2010) *Vogesella mureinivorans* sp. nov., a peptidoglycan-degrading bacterium from lake water. *Int J Syst Evol Microbiol* **60:** 2467–2472.

Jürgens, K., and Sala, M.M. (2000) Predation-mediated shifts in size distribution of microbial biomass and activity during detritus decomposition. *Oikos* **91:** 29–40.

Jürgens, K., Pernthaler, J., Schalla, S., and Amann, R. (1999)

Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. *Appl Environ Microbiol* **65:** 1241–1250.
Kämpfer, P. (1995) Physiological and chemotaxonomic characterization of filamentous bacteria belonging to the genus *Haliscomenobacter*. *Syst Appl Microbiol* **18:** 363–367.
Kang, H.J., Freeman, C., Park, S.S., and Chun, J. (2005) N-Acetylglucosaminidase activities in wetlands: a global survey. *Hydrobiologia* **532:** 103–110.
Kawasaki, N., and Benner, R. (2006) Bacterial release of dissolved organic matter during cell growth and decline: molecular origin and composition. *Limnol Oceanogr* **51:** 2170–2180.
King, G.M., and Berman, T. (1984) Potential effects of isotopic dilution on apparent respiration in C-14 heterotrophy experiments. *Mar Ecol Prog Ser* **19:** 175–180.
Kirchman, D. (2001) Measuring bacterial biomass production and growth rates from leucine incorporation in natural aquatic environments. *Methods Microbiol* **30:** 227–237.
Köllner, K.E., Carstens, D., Keller, E., Vazquez, F., Schubert, C.J., Zeyer, J., and Bürgmann, H. (2012) Bacterial chitin hydrolysis in two lakes with contrasting trophic statuses. *Appl Environ Microbiol* **78:** 695–704.
Kragelund, C., Levantesi, C., Borger, A., Thelen, K., Eikelboom, D., Tandoi, V., *et al.* (2008) Identity, abundance and ecophysiology of filamentous bacteria belonging to the Bacteroidetes present in activated sludge plants. *Microbiology* **154:** 886–894.
Lee, B.G., and Fisher, N.S. (1992) Decomposition and release of elements from zooplankton debris. *Mar Ecol Prog Ser* **88:** 117–128.
Middelboe, M., and Jørgensen, N.O.G. (2006) Viral lysis of bacteria: an important source of dissolved amino acids and cell wall compounds. *J Mar Biolog Assoc UK* **86:** 605–612.
Nagata, T., and Kirchman, D.L. (1992) Release of macromolecular organic-complexes by heterotrophic marine flagellates. *Mar Ecol Prog Ser* **83:** 233–240.
Nagata, T., and Kirchman, D.L. (2000) Bacterial mortality: a pathway for the formation of refractory DOM? In *Microbial Biosystems: New Frontiers – Proceedings of the 8th International Symposium on Microbial Ecology*. Bell, C.R., Brylinsky, M., and Johnson-Green, P. (eds). Halifax, NS, Canada: Atlantic Canada Society for Microbial Ecology, pp. 153–158.
Nagata, T., Meon, B., and Kirchman, D.L. (2003) Microbial degradation of peptidoglycan in seawater. *Limnol Oceanogr* **48:** 745–754.
Nedoma, J., Vrba, J., Hejzlar, J., Šimek, K., and Straskrabová, V. (1994) N-Acetylglucosamine dynamics in freshwarer environments – concentration of amino-sugars, extracellular enzyme-activities, and microbial uptake. *Limnol Oceanogr* **39:** 1088–1100.
Newton, R.J., Jones, S.E., Eiler, A., McMahon, K.D., and Bertilsson, S. (2011) A guide to the natural history of freshwater lake bacteria. *Microbiol Mol Biol Rev* **75:** 14–49.
Pernthaler, J. (2005) Predation on prokaryotes in the water column and its ecological implications. *Nat Rev Microbiol* **3:** 537–546.
Pernthaler, J., Posch, T., Šimek, K., Vrba, J., Pernthaler, A., Glöckner, F.O., *et al.* (2001) Predator-specific enrichment of actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture. *Appl Environ Microbiol* **67:** 2145–2155.
Pernthaler, J., Zöllner, E., Warnecke, F., and Jürgens, K. (2004) Bloom of filamentous bacteria in a mesotrophic lake: identity and potential controlling mechanism. *Appl Environ Microbiol* **70:** 6272–6281.
Posch, T., Franzoi, J., Prader, M., and Salcher, M.M. (2009) New image analysis tool to study biomass and morphotypes of three major bacterioplankton groups in an alpine lake. *Aquat Microb Ecol* **54:** 113–126.
Reasoner, D.J., and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Appl Environ Microbiol* **49:** 1–7.
Riemann, L., and Middelboe, M. (2002) Viral lysis of marine bacterioplankton: implications for organic matter cycling and bacterial clonal composition. *Ophelia* **56:** 57–68.
Rösel, S., and Grossart, H.P. (2012) Contrasting dynamics in activity and community composition of free-living and particle-associated bacteria in spring. *Aquat Microb Ecol* **66:** 169–181.
Salcher, M.M., Pernthaler, J., Psenner, R., and Posch, T. (2005) Succession of bacterial grazing defense mechanisms against protistan predators in an experimental microbial community. *Aquat Microb Ecol* **38:** 215–229.
Salcher, M.M., Pernthaler, J., and Posch, T. (2010) Spatiotemporal distribution and activity patterns of bacteria from three phylogenetic groups in an oligomesotrophic lake. *Limnol Oceanogr* **55:** 846–856.
Schauer, M., Jiang, J., and Hahn, M.W. (2006) Recurrent seasonal variations in abundance and composition of filamentous SOL cluster bacteria (Saprospiraceae, Bacteroidetes) in oligomesotrophic Lake Mondsee (Austria). *Appl Environ Microbiol* **72:** 4704–4712.
Sekar, R., Pernthaler, A., Pernthaler, J., Warnecke, F., Posch, T., and Amann, R. (2003) An improved protocol for quantification of freshwater Actinobacteria by fluorescence in situ hybridization. *Appl Environ Microbiol* **69:** 2928–2935.
Sherr, E., and Sherr, B.F. (1993) Preservation and storage of samples for enumeration of heterotrophic protist. In *Handbook of Methods in Aquatic Microbial Ecology*. Kemp, P., Sherr, E., Sherr, B.F., and Cole, J.J. (eds). Boca Raton, FL, USA: Lewis, pp. 207–212.
Šimek, K., Pernthaler, J., Weinbauer, M.G., Horňák, K., Dolan, J.R., Nedoma, J., *et al.* (2001) Changes in bacterial community composition and dynamics and viral mortality rates associated with enhanced flagellate grazing in a mesoeutrophic reservoir. *Appl Environ Microbiol* **67:** 2723–2733.
Šimek, K., Weinbauer, M.G., Horňák, K., Jezbera, J., Nedoma, J., and Dolan, J.R. (2007) Grazer and virus-induced mortality of bacterioplankton accelerates development of Flectobacillus populations in a freshwater community. *Environ Microbiol* **9:** 789–800.
Tang, K.W., Bickel, S.L., Dziallas, C., and Grossart, H.P. (2009) Microbial activities accompanying decomposition of cladoceran and copepod carcasses under different environmental conditions. *Aquat Microb Ecol* **57:** 89–100.
Tarao, M., Jezbera, J., and Hahn, M.W. (2009) Involvement

of cell surface structures in size-independent grazing resistance of freshwater Actinobacteria. *Appl Environ Microbiol* **75:** 4720–4726.

Tranvik, L. (1994) Colloidal and dissolved organic matter excreted by a mixotrophic flagellate during bacterivory and autotrophy. *Appl Environ Microbiol* **60:** 1884–1888.

van Veen, W.L., Van Der Kooij, D., Geuze, E.C.W., and Van Der Vlies, A.W. (1973) Investigations on the sheathed bacterium *Haliscomenobacter hydrossis* gen.n., sp.n., isolated from activated sludge. *Antonie Van Leeuwenhoek* **39:** 207–216.

Velicer, G.J. (2003) Social strife in the microbial world. *Trends Microbiol* **11:** 330–337.

Vrba, J., Šimek, K., Nedoma, J., and Hartman, P. (1993) 4-Methylumbelliferyl-β-N-Acetylglucosaminide hydrolysis by a high-affinity enzyme, a putative marker of protozoan bacterivory. *Appl Environ Microbiol* **59:** 3091–3101.

Wang, S.L., and Chang, W.T. (1997) Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. *Appl Environ Microbiol* **63:** 380–386.

Warnecke, F., Sommaruga, R., Sekar, R., Hofer, J.S., and Pernthaler, J. (2005) Abundances, identity, and growth state of Actinobacteria in mountain lakes of different UV transparency. *Appl Environ Microbiol* **71:** 5551–5559.

Weinbauer, M.G., Horňák, K., Jezbera, J., Nedoma, J., Dolan, J.R., and Šimek, K. (2007) Synergistic and antagonistic effects of viral lysis and protistan grazing on bacterial biomass, production and diversity. *Environ Microbiol* **9:** 777–788.

Weisse, T., Müller, H., Pintocoelho, R.M., Schweizer, A., Springmann, D., and Baldringer, G. (1990) Response of the microbial loop to the phytoplankton spring bloom in a large prealpine lake. *Limnol Oceanogr* **35:** 781–794.

Wurzbacher, C.M., Barlocher, F., and Grossart, H.P. (2010) Fungi in lake ecosystems. *Aquat Microb Ecol* **59:** 125–149.

Zeder, M., and Pernthaler, J. (2009) Multispot live-image autofocusing for high-throughput microscopy of fluorescently stained bacteria. *Cytometry A* **75A:** 781–788.

ARTICLE III

ECKERT EM & PERNTHALER J (SUBMITTED) BACTERIAL EPIBIONTS OF DAPHNIA ARE A POTENTIAL ROUTE FOR THE EFFICIENT TRANSFER OF DISSOLVED ORGANIC CARBON WITHIN FRESHWATER FOOD WEBS.

Bacterial epibionts of *Daphnia* are a potential route for the efficient transfer of dissolved organic carbon within freshwater food webs

Short title: Substrate uptake by epibionts of Daphnia

Ester M. Eckert & Jakob Pernthaler*

Limnological Station, Institute of Plant Biology, University of Zurich, Seestrasse 187, 8802 Kilchberg, Switzerland

*corresponding author: Phone: 0041 44 634 9210; Fax: 0041 44 634 92 25; Email: pernthaler@limnol.uzh.ch

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Abstract

The identification of interacting species and elucidation of their mode of interaction may be crucial to understand ecosystem-level processes. We analysed the activity and identity of bacterial epibionts in cultures of *Daphnia galeata* and of natural daphnid populations. Epibiotic bacteria incorporated considerable amounts of dissolved organic carbon (DOC), as estimated via uptake of tritiated Leucine: three times more tracer was consumed by microbes on a single *Daphnia* than in a mL of lake water. However, there was virtually no incorporation if daphnids were anesthetized, suggesting that their filtration activity was essential for this process. Microbial DOC uptake could predominantly be assigned to microbes that were located on the filter combs of daphnids, where the passage of water would ensure a continuously high DOC supply. Most of these bacteria were *Betaproteobacteria* from the genus *Limnohabitans*. Specifically, we identified a monophyletic cluster harbouring *L. planktonicus* that encompassed sequence types from *D. galeata* cultures, from the gut of *D. magna,* and from daphnids of Lake Zurich. Our results suggest that the epibiotic growth of bacteria related to *Limnohabitans* on *Daphnia* spp. may be a widespread and rather common phenomenon. Moreover, most of the observed DOC flux to *Daphnia* in fact does not seem to be associated with the crustacean biomass itself but with its epibiotic microflora. The unexplored physical association of daphnids with heterotrophic bacteria may have considerable implications for our understanding of carbon transfer in freshwater food webs, i.e., a trophic ‘shortcut’ between microbial DOC uptake and predation by fish.

Introduction

In order to accurately estimate element fluxes or predict ecosystem responses it is essential to understand food web architecture (1). Unexplored trophic links may considerably alter food web structure, and can, once investigated, substantially change our understanding of ecosystem carbon metabolism (2, 3). Therefore, interacting species and their mode of interaction need to be identified for more accurate assumptions about ecosystem functionality and stability (4).

The lack of information about the physical interactions between heterotrophic bacteria and zooplankton, such as the crustacean genus *Daphnia*, may neglect an important aspect of freshwater food webs (5). Indeed, little is known about the microbial communities associated with healthy *Daphnia*, as opposed to the plethora of research about the respective roles of either bacterio- or zooplankton within aquatic food webs, or about microbial parasites of *Daphnia* (e.g. (6-8)). Recent studies, however, point to other system-relevant associations between bacteria and *Daphnia,* e.g. the transfer of microbes from lower to higher water layers via attachment-detachment processes (9). The gut microflora of *Daphnia magna* was reported to be dominated by members of the genus *Limnohabitans* (10), i.e., by common inhabitants of the pelagic zone of freshwater epilimnia (11) that typically co-occur with phytoplankton (12). High bacterial diversity, including phylotypes related to *Limnohabitans,* was also found when analysing prokaryotic sequences from metagenomic data of *Daphnia* spp. (13).

While algae are generally regarded as the main food for daphnids, heterotrophic bacteria are considered less important (14-16). However, some bacteria, notably filamentous morphotypes, are also consumed by *Daphnia* sp., which in turn may directly affect bacterial community structure at least during particular seasons (17-19). In addition, it is conceivable that the associations of zooplankton with heterotrophic bacteria may also have substantial implications for biogeochemical processes such as the transfer of carbon through the food web: while daphnids feed on organic carbon from the particulate fraction (20), the attached bacteria would likely consume dissolved organic carbon (DOC). Apart from a single more recent report (21), uptake of DOC by *Daphnia* (or by their epibionts) has been addressed by studies dating from the beginning of the last century (e.g., (22, 23)). This largely unexplored trophic link might, however, be of great relevance for lake carbon cycling, e.g., in the context of the much debated question to which extend

internal primary production or terrestrial carbon sources support freshwater ecosystems (e.g. (24-26)). In such studies the biomass of homogenised daphnids is proportionally assigned to allochthonous or autochthonous sources -by analysis of the isotopic ratios of carbon atoms- in order to model the fluxes of organic carbon through the food web. The zooplankton epibionts are considered as part of the *Daphnia* biomass; however, their specific metabolic abilities (i.e, consumption of DOC) might considerably affect the interpretation of such assessments.

We studied the uptake of dissolved leucine by *Daphnia galeata* or their epibionts to gain first insight into the importance of this trophic link, and we compared it to their ingestion of leucine incorporating planktonic microbes. Furthermore, we localised and identified a prominent genus of microbial epibionts responsible for this uptake on cultured *D. galeata* as well as on daphnids from mixed natural populations in a lake (Lake Zurich, Switzerland). Using leucine and N-acetyl-D-glucosamine (NAG) as model substrates we then assessed the metabolic activity of *Daphnia* epibionts in Lake Zurich.

Results & Discussion

Uptake of dissolved leucine by epibionts of Daphnia galeata

We labelled a natural microbial community with tritiated leucine (Leu), and let *D. galeata* feed on it for 1h (experimental setup: Fig 1). The amount of radiolabel in *D. galeata* due to their incorporation of both, dissolved Leu and microbes (Raw water-treatment [Rw], Fig 1) was compared to the uptake of dissolved Leu only, in a treatment where daphnids were placed into the sample after removing the labelled microbial cells by filtration (treatment Filtrate [F], Fig 1). The amount incorporated by additionally feeding on microorganisms did not significantly exceed the uptake of substrate from the dissolved fraction only (Fig 2), indicating that Leu-incorporating heterotrophic microbes were of minor importance as a food source for daphnids (14-16). Substantial amounts of radioactivity were detected in daphnids maintained on the filtrate, suggesting uptake of dissolved substrate by the animals (Fig 2): Free living bacteria in 1 mL of water incorporated 0.1 ± 0.04 pmol Leu h^{-1} whereas three times higher uptake rates (0.3 ± 0.04 pmol h^{-1}) were observed per individual daphnid (Fig 2). The level of incorporation of dissolved Leu in this study far exceeds the previously reported uptake of custom labelled algal exudates by daphnids (21). This might be ascribed to methodological issues, e.g., a low labelling efficiency of the exudates.

To further explore the notion that Leu was readily taken up by epibiotic bacteria, labelled *D. galeata* individuals were dissected and the separated pieces were overlaid with a photographic emulsion, to microscopically localise the deposition of radioactivity on the animal and in bacterial cells by microautoradiography (MAR) and fluorescence *in situ* hybridization (FISH) (27). Strongest labelling was detected around the trunk limbs; particularly on the setae and appendages of trunk limbs 3 and 4 (TL3&4, Fig 3). Most uptake on these body surfaces could be assigned to single bacterial cells (Fig 3). The setae and setula of trunk limb 3 and 4 serve as food capturing filter sieves (e.g.(28)), and daphnids incessantly filter water through these structures. Besides protecting from protistan grazers and abiotic stressors (5, 29), a steady supply of organic carbon and nutrients may render the filter apparatus an ideal habitat for epibiotic bacteria. This interpretation is further supported by our finding that almost no label was incorporated when daphnids were anesthetised before being placed in the filtrate (treatment Anesthetised [A], Fig 1). Thus, active filtration by the animals seemed to be a prerequisite for substrate uptake by the

epibionts. Furthermore, the proportions of incorporated radioactivity in the external body parts of dissected daphnids were between 4 and >80 times higher (mean, 29.5) then in the colons. Epibiotic bacteria have also been described from the feeding appendages of marine copepods, as well as on setae of other crustaceans such as the deep sea Yeti crab, *Kiwa hirsuta* (30, 31). It should be noted that approximately 20% of the filter combs in 20 analysed daphnids were nearly uncolonized by bacteria. This might be due to moulting processes, which have been shown to reduce the parasite loads on daphnids (32) and thus likely also affect the densities of other epibionts.

Active epibionts affiliated with Limnohabitans

Previous studies centred on the identity of *Daphnia* associated microbes hint at the importance of *Betaproteobacteria,* in particular of bacteria related to the genus *Limnohabitans* (10, 13, 33). Therefore, we performed FISH on dissected individuals of *D. galeata,* using an oligonucleotide probe for a phylogenetic cluster (*Lhb*) that includes the type strains *L. planktonicus* and *L. parvus* (34). A large proportion of the epibionts on the *D. galeata* filtration apparatus were affiliated with *Lhb* (Fig 3), whereas <0.5% of cells in the surrounding cultivation water were hybridised with this probe. Most of the epibiotic *Lhb* bacteria showed visible incorporation of Leu (Fig 3), as detected by MAR-FISH (27). Planktonic *Limnohabitans* spp. are known to readily incorporate this substrate (35, 36). Cultures of *L. planktonicus* have, moreover, been shown to profit from the presence of algae, which has been ascribed to their utilization of algal exudates (12). In addition to the advantageous supply of such fresh DOC to *Limnohabitans* spp. on *Daphnia* filtration combs by the filtration activity itself, it is also conceivable that these bacteria might further profit from the products of ‘sloppy feeding’, i.e. organic compounds released by the physical breaking of algal cells (e.g., amino acids (37)).

FISH on segments of various dissected *daphnids* from Lake Zurich confirmed the presence of epibiotic *Lhb* bacteria on natural zooplankton populations (Fig. 3). While only up to 3% of the heterotrophic bacteria in lake water were affiliated with *Lhb* (Fig 4), filter combs of *daphnids* were typically covered by bacteria from this genus that were, moreover, visibly incorporating Leu. In addition, there were clear morphological differences between planktonic *Lhb* and those associated with

daphnids, e.g., only the latter formed filamentous morphotypes (Fig 3). Similar to observations in cultures of *D. galeata,* some filtration combs of daphnids in Lake Zurich were also virtually free of bacteria (in 3 out of 17 analysed individuals). In addition, other, unidentified bacteria were occasionally found to dominate on the filter seata (Fig 3).

Indications for a core group of epibiontic Limnohabitans *sp.*

To investigate the phylogenetic relationship of *Lhb* bacteria on daphnids from various sources we constructed 16S ribosomal DNA clone libraries using the sequence of the *Lhb* probe as a forward primer together with a general bacterial primer. By this we identified bacteria associated with *D. galeata* and Lake Zurich daphnids as well as from the respective surrounding water.

No sequence obtained from the cultivation water of *D. galeata* were from the genus *Limnohabitans*. In contrast, *Lhb* sequences were obtained from water samples of Lake Zurich. These sequences were, however, considerably different from the ones retrieved from daphnids (Fig S1). These findings indicate a degree of specificity of the associations between *Lhb* bacteria and their host (38).

Highest similarity was detected between *Lhb* phylotypes retrieved from daphnids from Lake Zurich and from the cultured *D. galeata* (Fig S1). The sequences clustered in two shared operational taxonomic units (OTUs, 99% identity level), as well as forming one specific OTU per source population. One of the shared clusters also included sequences retrieved from the digestive tract of *D. magna* (10) and the type strain *L. planktonicus* (Fig S1 (39)). It thus seems that there are core phylotypes closely affiliated with *L. planktonicus* that are commonly associated with different species of daphnids from various habitats, as well as a more variable set of other microbiota (9). *L. planktonicus* was originally isolated from pelagic samples of a freshwater reservoir (40). Subsequent analysis, however, revealed, that this species is not common in the pelagic zone of lacustrine waters (41). It is thus possible that these bacteria in fact predominately inhabit an epibiotic niche. Epibiosis may however not be the exclusive place of occurrence for a particular genotype: The human pathogen *Vibrio cholera* is present in high abundances in the mouth area of marine copepods, but these bacteria are also found free living in costal marine waters, albeit at low densities (42-44). Thus, *V. cholera* may be part of the ‘rare

biosphere' within pelagic communities while abundant on zooplankton. A similar occurrence pattern might be hypothesized for bacteria related to *L. planktonicus*.

Interestingly, closely related *Lhb* genotypes were found on the filtration combs of *D. galeata* and in the *D. magna* digestive tract. A possible explanation lies in the feeding physiology of *Daphnia*, i.e. part of the *Lhb* population on the filtration apparatus might be ingested and transported into the digestive system. Freshwater bacteria have been observed to pass the gut of daphnids alive (45). Whether or not *Lhb* bacteria play a role in the digestion processes within the colon of the animals remains to be explored, as well as the mode of interaction between the Daphnids and *Lhb*-epibionts on the filter combs. It should be emphasized that dead *D. galeata* were never found to be inhabited by *Lhb* bacteria (*n=6 inspected individuals*), as has already been observed for *Daphnia magna* (10). This may indicate that the interaction between *Lhb* bacteria and the animals is not of a pathogenic nature.

Activity of Daphnia *epibionts in Lake Zurich: implication for food webs*

In order to assess the *in situ* relevance of DOC uptake by daphnids (or their epibionts, respectively) we sampled Lake Zurich throughout April and May, starting at the onset of the clear water phase after the spring phytoplankton bloom. *Daphnia* reached abundances of up to 13 adult and 28 juvenile individuals l^{-1} (Fig 4), and were dominated by members of the *D. longispina* species complex (*D. galeata, cucullata, longispina*, and hybrids; data: water supply Zurich). The uptake of dissolved Leu by daphnids and by bacterioplankton was determined on four dates (Fig 4 & 5). These experiments were in principle designed as described for the *D. galeata* cultures, with minor modifications (see Fig S2 and below). On three of the four dates we additionally tested for uptake of *N*-acetylglucosamine (NAG) (Fig 5), a substrate that is not incorporated by *Lhb* (46). High uptake rates of both, dissolved Leu and NAG were detected, albeit with large variations between dates (Fig 5). Interestingly, the proportional amount of NAG taken up by the daphnids via consumption of labelled bacteria (normalized to the labelled bacterioplankton community, i.e., (Rw-F)/B) was consistently higher by about fivefold then of Leu, indicating that *Daphnia* preferentially fed on NAG- rather than Leu-incorporating bacteria. This might be explained by the high NAG uptake rates of large filamentous bacteria that are more likely to be grazed by daphnids than, e.g. small rod-shaped cells specialized for Leu incorporation (17, 18, 47, 48). Alternatively, there might be NAG uptake by some

226 phytoplankton species that are consumed by daphnids (49). Thus the transfer mode
227 of DOC to daphnids may in fact differ for individual organic compounds: While the
228 direct incorporation of heterotrophic bacteria seemed unimportant for Leu uptake of
229 zooplankton, their foraging on NAG-labelled microbes was of greater relevance.

230 The observed uptake of both, NAG and Leu by daphnids incubated in
231 bacteria-free filtrates is evidence for a high, temporarily variable DOC incorporation *in*
232 *situ*, likely mediated via the attached bacterial flora. This is suggested by the high
233 proportion of tracer incorporation on the external body surfaces, as compared to the
234 digestive tract. If extrapolated to the total daphnid population in lake water, the
235 incorporation amounted to up to 8% of the Leu and nearly 10% of the NAG uptake by
236 the bacterioplankton. While this proportion may seem small at a first glance, such
237 direct transfer of low molecular weight DOC to zooplankton may nevertheless be of
238 considerable importance. The positive effect of *Daphnia* on the size of fish
239 populations in lakes is mainly attributed to the fact that *Daphnia* feed on primary
240 producers and are in turn consumed by fish i.e. the cascade algae-daphnia-fish is
241 considered to be highly efficient because of its shortness (50, 51). Similarly, the
242 prokaryotic epibionts on daphnids may form a more direct link between microbial
243 DOC uptake and fish predation. Such a shortcut would circumvent the passage of
244 substrates through intermediate levels of the microbial food web (heterotrophic nano-
245 flagellates, ciliates), thereby avoiding the significant respiration losses associated
246 with these trophic transition (50, 52). This suggests that zooplankton epibionts such
247 as *Limnohabitans* sp. might play a disproportionally important role for the transfer of
248 DOC from both, autochthonous and terrestrial sources to the top trophic levels in
249 lacustrine ecosystems.

250

Materials and Methods

Sampling and sample preparation

Lake Zurich was sampled weekly at around 10 am between April 26 and May 24 2012 (coordinates 47°31' N, 8°58' E). Chlorophyll *a* and temperature were measured using a multiple-wavelength probe (TS-16–12 fluoroprobe, bbe Moldaenke GmbH, Kronshagen, Germany) and a multi-parameter probe (6600 multi-parameter, water quality monitoring, YSI incorp., Yellow Springs, OH, USA), respectively. The sample taken on April 26 was from the depth of maximum Chl *a* (8m), while later samples were collected from 5m depth because of the onset of the clear water phase (Fig 4). Zooplankton was collected using a Ruttner sampler, concentrated with a 40µm net from a volume of 5L and directly fixed with formaldehyde (FA, final concentration, 4%) for determination of abundances. Live daphnids were collected using the same device and transported to the laboratory in a clean jar. A third set of daphnids were immediately anaesthetised with carbon dioxide enriched water for later experiments. The daphnids were kept in a laboratory incubator at *in situ* temperature for a maximum of 3h prior to the experiments.

Fifty mL of lake water were fixed with FA (final concentration, 1%) for the analysis of total bacterial abundances and the proportions of *Lhb* bacteria. Subsamples of 4mL were filtered onto white polycarbonate membrane filters (type GTTP, 45 mm diameter, 0.2 mm pore size, Millipore, Billerica, MA, USA) for the counting of *Lhb* bacteria. The remaining sample was stored at 4°C for flow cytometric determination of total cell numbers.

Tracer Experiments

In Experiment I (Fig 1) *Daphnia galeata* females were kept in sterile (0.2µm prefiltered and autoclaved) lake water (sLW) and fed with *Scenedesmus subspicatus* approximately every second day. Adult individuals were washed three times with sLW, and 6 sets of 4 individuals were transferred to 5mL Erlenmeyer flasks containing 1mL of sLW. Twelve additional individuals were anaesthetised in commercial carbon dioxide enriched water, washed twice in sLW and 3 sets of 4 individuals were placed in 5mL Erlenmeyer flasks containing 1mL of sLW. Daphnids were acclimatised for 1h in the dark at 20°C before tracer addition.

Nine sets of 30µm prefiltered lake water (1mL each) were incubated for 1h with 10nM of tritium labelled Leu (specific activity: 120 Ci $mmol^{-1}$) or NAG (specific

284 activity: 60 Ci mmol^{-1}, American Radiolabeled Chemicals, Inc., St. Louis, Mo, USA) to
285 label the microbial community. The labelled communities were then distributed to
286 triplicate Erlenmeyer flasks containing live daphnids (treatment Rw) or anesthetised
287 daphnids (treatment A), respectively. The third set of labelled microbial communities
288 was filtered onto nitrocellulose membrane filters (type GSWP, 45 mm diameter,
289 0.22µm pore size, Millipore, Billerica, MA, USA) to determine microbial incorporation
290 of the tracer. The filtrate was also collected and added to triplicate Erlenmeyer flasks
291 each containing four daphnids (treatment F).
292 Daphnids were incubated for 1h at 20°C on a laboratory rocker (10 rmp over a
293 tilt angle of ±11°). Thereafter, all daphnids were individually picked and anesthetized
294 in carbon dioxide rich water. Three individuals from each Erlenmeyer were then
295 transferred to separate scintillation vials with 500µl Soluene350 (Perkin Elmer Inc.,
296 San Jose, CA, USA) to solubilize the tissue and incubated at 50°C for approximately
297 6h. For one experiment six *D. galeata* individuals were dissected after labelling and
298 colons and the outer carapaces were separated for uptake measurements. When the
299 daphnids were dissolved 0.5mL of scintillation cocktail (Rotiszint eco plus, Carl Roth
300 GmbH, Karlsruhe, Germany) was added and radioactivity was measured in a
301 scintillation counter (*n=9* for each treatment and date). One daphnid from each
302 treatment and date was fixed with EtOH, placed on a cover slip and dissected under
303 a binocular microscope for later FISH and MAR-FISH analysis (see below).
304 Experiment II (Fig S2) was essentially performed as described for Experiment
305 I, except that daphnids were collected from Lake Zurich, and the microbial
306 communities in the A- and F-treatments were removed already before addition of the
307 radioactive tracer to assess the total potential uptake of *Daphnia sp.* epibionts.
308 Separate triplicate sets of water samples (1mL) were used to determine tracer uptake
309 by the pelagic bacterial assemblages. Finally, all incubations for Experiment II were
310 performed at *in situ* (lake water) temperatures.
311
312 *Staining and microscopic analysis*
313 Dissected Daphnids on the cover slips were overlaid with a drop of 0.1% low
314 melting point agarose and dried at 45°C. The cover slip was incubated in 90% EtOH
315 for 45min to fix the bacterial community. FISH and MAR-FISH with probe R-BT065
316 (34) (targeting bacteria affiliated with *Lhb*) were essentially conducted as described
317 before (27, 53) albeit on the cover slips, and predigestion was reduced to a lysozyme

318 treatment of 20min only. Microscopic imaging of the filtration apparatus was done on
319 an inverse confocal laser scanning microscope (CLSM Leica SP2, Leica
320 Microsystems, Wetzlar, Germany) at the Centre for Microscopy and Image Analysis
321 of the University of Zurich. Images were further processed with the software package
322 Imaris x64 version 7.5.2 (Bitplane AG, Zurich, Switzerland) and arranged using
323 Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA).

324 *Phylogenetic analysis*

325 Four 16S rRNA gene clone libraries were constructed to analyse the phylogenetic
326 composition of *Lhb* bacteria on daphnids from Lake Zurich, on cultured *D. galeata*
327 and in the surrounding media (Lake Zurich water from 5m depth and *D. galeata*
328 cultivation medium). For this purpose, daphnids and water samples from Lake Zurich
329 were obtained on March 31. All daphnids were washed 3 times in sterile, UV-treated
330 deionized water. DNA was isolated from approximately 13-16 daphnids or 25mL of
331 filtered water using the PowerSoil DNA isolation kit (MO BIO Laboratories, Inc.,
332 Carlsbad, CA, USA). 16S rRNA gene sequences affiliated with *Lhb* were amplified
333 using the R-Bt065 oligonucleotide (34) as a forward primer and a general bacterial
334 reverse primer (54) (annealing temperature: 57°, 30s; GoTaq Green Master Mix,
335 Promega Co., Madison WI, USA). The purified PCR products were cloned into
336 competent *Escherichia coli* (pGEM-T Easy Vectors, Promega Co.). The clones were
337 screened for inserts and positive clones were sequenced. The sequences were
338 assembled using DNA Baser Sequence Assembler (Heracle BioSoft S.R.L., Pitesti,
339 Romania), aligned with the SINA web aligner (55) and merged into the SILVA SSU
340 reference database 110 using the software package ARB (56). Uchime (57) was
341 used to exclude chimeric sequences. Bootstrapped Maximum Likelihood trees (1000
342 repetitions, (58)) were calculated that comprised the sequences from this study,
343 *Limnohabitans* sp. isolates as published in Kasalický et al (59), and *Limnohabitans*
344 sequences obtained from *D. magna* gut analysis (10). The same set of sequences
345 was clustered into OTUs (99% identity) using Mothur (60), and the OTU clustering
346 pattern was compared to the results of the phylogenetic analysis. All sequences are
347 deposited in the EMBL database with accession numbers HF96498 – HF968621.
348

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References

1. *Bascompte J (2010) Structure and Dynamics of Ecological Networks. Science 329(5993):765-766*
2. *Kuwae T, et al. (2012) Variable and complex food web structures revealed by exploring missing trophic links between birds and biofilm. Ecol Lett 15(4):347-356*
3. *Corno G, Villiger J, Pernthaler J (2012) Coaggregation in a microbial predator–prey system affects competition and trophic transfer efficiency. Ecology 94(4):870-881*
4. *Thébault E, Fontaine C (2010) Stability of Ecological Communities and the Architecture of Mutualistic and Trophic Networks. Science 329(5993):853-856*
5. *Tang KW, Turk V, Grossart HP (2010) Linkage between crustacean zooplankton and aquatic bacteria. Aquat Microb Ecol 61(3):261-277*
6. *Miner BE, De Meester L, Pfrender ME, Lampert W, Hairston NG (2012) Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proc R Soc B-Biol Sci 279(1735):1873-1882*
7. *Newton RJ, Jones SE, Eiler A, McMahon KD, Bertilsson S (2011) A Guide to the Natural History of Freshwater Lake Bacteria. Microbiol Mol Biol Rev 75(1):14-49*
8. *Ebert D (2008) Host-parasite coevolution: Insights from the Daphnia-parasite model system. Curr Opin Microbiol 11(3):290-301*
9. *Grossart HP, Dziallas C, Leunert F, Tang KW (2010) Bacteria dispersal by hitchhiking on zooplankton. Proc Natl Acad Sci USA 107(26):11959-11964*
10. *Freese H, Schink B (2011) Composition and Stability of the Microbial Community inside the Digestive Tract of the Aquatic Crustacean Daphnia magna. Microb Ecol 62(4):882-894*

11. *Šimek K, et al. (2005) Influence of top-down and bottom-up manipulations on the R-BT065 subcluster of beta-proteobacteria, an abundant group in bacterioplankton of a freshwater reservoir. Appl Environ Microbiol 71(5):2381-2390*
12. *Šimek K, Kasalický V, Zapomelova E, Horňák K (2011) Alga-Derived Substrates Select for Distinct Betaproteobacterial Lineages and Contribute to Niche Separation in Limnohabitans Strains. Appl Environ Microbiol 77(20):7307-7315*
13. *Qi WH, Nong G, Preston JF, Ben-Ami F, Ebert D (2009) Comparative metagenomics of Daphnia symbionts. BMC Genomics 10(172):1471-2164*
14. *Nagata T, Okamoto K (1988) Filtering rates on natural bacteria by Daphnia longispina and Eodiaptomus japonicus in Lake Biwa J Plankton Res 10(4):835-850*
15. *Peterson BJ, Hobbie JE, Haney JF (1978) Daphnia grazing on natural bacterial. Limnol Oceanogr 23(5):1039-1044*
16. *Martin-Creuzburg D, Beck B, Freese HM (2011) Food quality of heterotrophic bacteria for Daphnia magna: evidence for a limitation by sterols. FEMS Microbiol Ecol 76(3):592-601*
17. *Langenheder S, Jürgens K (2001) Regulation of bacterial biomass and community structure by metazoan and protozoan predation. Limnol Oceanogr 46(1):121-134*
18. *Pernthaler J, Zöllner E, Warnecke F, Jürgens K (2004) Bloom of filamentous bacteria in a mesotrophic lake: Identity and potential controlling mechanism. Appl Environ Microbiol 70(10):6272-6281*
19. *Taipale S, Kankaala P, Tiirola M, Jones RI (2008) Whole-lake dissolved inorganic (13)C additions reveal seasonal shifts in zooplankton diet. Ecology 89(2):463-474*
20. *Cole JJ, et al. (2006) Differential support of lake food webs by three types of terrestrial organic carbon. Ecol Lett 9(5):558-568*
21. *Speas DW, Duffy WG (1998) Uptake of dissolved organic carbon (DOC) by Daphnia pulex. J Freshw Ecol 13(4):457-463*
22. *Kerb H (1911) Über den Nährwert der im Wasser gelösten Stoffe. Internationale Revue der gesamten Hydrobiologie und Hydrographie 3(5-6):496-505*
23. *Krogh A (1930) Über die Bedeutung von gelösten organischen Substanzen bei der Ernährung von Wassertieren. Zeitschrift für vergleichende Physiologie 12:668-681*
24. *Grey J, Jones RI, Sleep D (2001) Seasonal changes in the importance of the source of organic matter to the diet of zooplankton in Loch Ness, as indicated by stable isotope analysis. Limnol Oceanogr 46(3):505-513*
25. *Pace ML, et al. (2004) Whole-lake carbon-13 additions reveal terrestrial support of aquatic food webs. Nature 427(6971):240-243*
26. *Brett MT, Kainz MJ, Taipale SJ, Seshan H (2009) Phytoplankton, not allochthonous carbon, sustains herbivorous zooplankton production. Proc Natl Acad Sci USA 106(50):21197-21201*
27. *Alonso C, Pernthaler J (2005) Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. Appl Environ Microbiol 71(4):1709-1716*
28. *Fryer G (1991) Functional Morphology and the Adaptive Radiation of the Daphniidae (Branchiopoda: Anomopoda). Phil Trans R Soc Lond B 331(1259):1-99*
29. *Tang KW, Dziallas C, Grossart HP (2011) Zooplankton and aggregates as refuge for aquatic bacteria: protection from UV, heat and ozone stresses used for water treatment. Environ Microbiol 13(2):378-390*
30. *Carman KR, Dobbs FC (1997) Epibiotic microorganisms on copepods and other marine crustaceans. Microsc Res Tech 37(2):116-135*

436 *31. Goffredi SK, Jones WJ, Erhlich H, Springer A, Vrijenhoek RC (2008) Epibiotic*
437 *bacteria associated with the recently discovered Yeti crab, Kiwa hirsuta. Environ*
438 *Microbiol 10(10):2623-2634*
439 *32. Duneau D, Ebert D (2012) The role of moulting in parasite defence. Proc R Soc B-*
440 *Biol Sci 279(1740):3049-3054*
441 *33. Peter H, Sommaruga R (2008) An evaluation of methods to study the gut bacterial*
442 *community composition of freshwater zooplankton. J Plankton Res 30(9):997-1006*
443 *34. Šimek K, et al. (2001) Changes in bacterial community composition and dynamics*
444 *and viral mortality rates associated with enhanced flagellate grazing in a*
445 *mesoeutrophic reservoir. Appl Environ Microbiol 67(6):2723-2733*
446 *35. Horňák K, Jezbera J, Nedoma J, Gasol JM, Šimek K (2006) Effects of resource*
447 *availability and bacterivory on leucine incorporation in different groups of*
448 *freshwater bacterioplankton, assessed using microautoradiography. Aquat Microb*
449 *Ecol 45(3):277-289*
450 *36. Salcher MM, Posch T, Pernthaler J (2012) In situ substrate preferences of abundant*
451 *bacterioplankton populations in a prealpine freshwater lake. ISME J 13(10):162*
452 *37. Riemann B, Jørgensen NOG, Lampert W, Fuhrman JA (1986) Zooplankton Induced*
453 *Changes in Dissolved Free Amino Acids and in Production Rates of Freshwater*
454 *Bacteria. Microb Ecol 12(3):247-258*
455 *38. Wahl M, Goecke F, Labes A, Dobretsov S, Weinberger F (2012) The second skin:*
456 *Ecological role of epibiotic biofilms on marine organisms. Frontiers in Microbiology*
457 *3(292):1-21*
458 *39. Hahn MW, et al. (2010) Limnohabitans curvus gen. nov., sp nov., a planktonic*
459 *bacterium isolated from a freshwater lake. Int J Syst Evol Microbiol 60:1358-1365*
460 *40. Kasalicky V, Jezbera J, Simek K, Hahn MW (2010) Limnohabitans planktonicus sp.*
461 *nov. and Limnohabitans parvus sp. nov., planktonic betaproteobacteria isolated*
462 *from a freshwater reservoir, and emended description of the genus Limnohabitans.*
463 *Int J Syst Evol Microbiol 60(Pt 12):2710-2714*
464 *41. Jezbera J, Jezberová J, Kasalický V, Šimek K, Hahn MW (2013) Patterns of*
465 *Limnohabitans Microdiversity across a Large Set of Freshwater Habitats as Revealed*
466 *by Reverse Line Blot Hybridization. PLoS One 8(3):e58527*
467 *42. Huq A, et al. (1983) Ecological relationships between Vibrio cholerae and*
468 *planktonic crustacean copepods. Appl Environ Microbiol 45(1):275-283*
469 *43. Cottingham KL, Chiavelli DA, Taylor RK (2003) Environmental microbe and human*
470 *pathogen: the ecology and microbiology of Vibrio cholerae. Frontiers in Ecology*
471 *and the Environment 1(2):80-86*
472 *44. Heidelberg JF, Heidelberg KB, Colwell RR (2002) Seasonality of Chesapeake Bay*
473 *bacterioplankton species. Appl Environ Microbiol 68(11):5488-5497*
474 *45. King CH, Sanders RW, Shotts EB, Porter KG (1991) Differential survival of bacteria*
475 *ingested by zooplankton from a stratified eutrophic lake. Limnol Oceanogr*
476 *36(5):829-845*
477 *46. Eckert EM, Salcher MM, Posch T, Eugster B, Pernthaler J (2012) Rapid successions*
478 *affect microbial N-acetyl-glucosamine uptake patterns during a lacustrine spring*
479 *phytoplankton bloom. Environ Microbiol 14(3):794-806*
480 *47. Kragelund C, et al. (2008) Identity, abundance and ecophysiology of filamentous*
481 *bacteria belonging to the Bacteroidetes present in activated sludge plants.*
482 *Microbiology-(UK) 154:886-894*

483 *48. Eckert EM, Baumgartner M, Huber IM, Pernthaler J (2013) Grazing resistant*
484 *freshwater bacteria profit from chitin and cell-wall-derived organic carbon. Environ*
485 *Microbiol:doi: 10.1111/1462-2920.12083*
486 *49. Nedoma J, Vrba J, Hejzlar J, Šimek K, Straskrabová V (1994) N-Acetylglucosamine*
487 *Dynamics in Fresh-Warer Environments - Concentration of Amino-Sugars,*
488 *Extracellular Enzyme-Activities, and Microbial Uptake. Limnol Oceanogr 39(5):1088-*
489 *1100*
490 *50. Stockner JG, Shortreed KS (1989) Algal picoplankton production and contribution*
491 *to food-webs in oligotrophic British Columbia lakes. Hydrobiologia 173(2):151-166*
492 *51. Stockner J, Porter K (1988) Microbial Food Webs in Freshwater Planktonic*
493 *Ecosystems. Complex Interactions in Lake Communities, ed Carpenter S (Springer*
494 *New York), pp 69-83.*
495 *52. Pomeroy L, Wiebe W (1988) Energetics of microbial food webs. Hydrobiologia*
496 *159(1):7-18*
497 *53. Pernthaler A, Pernthaler J, Amann R (2002) Fluorescence in situ hybridization and*
498 *catalyzed reporter deposition for the identification of marine bacteria. Appl Environ*
499 *Microbiol 68(6):3094-3101*
500 *54. Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic relationships of*
501 *Thiomicrospira species and their identification in deep-sea hydrothermal vent*
502 *samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch*
503 *Microbiol 164(3):165-172*
504 *55. Pruesse E, et al. (2007) SILVA: a comprehensive online resource for quality checked*
505 *and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res*
506 *35(21):7188-7196*
507 *56. Ludwig W, et al. (2004) ARB: a software environment for sequence data. Nucleic*
508 *Acids Res 32(4):1363-1371*
509 *57. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves*
510 *sensitivity and speed of chimera detection. Bioinformatics 27(16):2194-2200*
511 *58. Stamatakis A, Hoover P, Rougemont J (2008) A Rapid Bootstrap Algorithm for the*
512 *RAxML Web Servers. Syst Biol 57(5):758-771*
513 *59. Kasalický V, Jezbera J, Hahn MW, Šimek K (2013) The Diversity of the*
514 *Limnohabitans Genus, an Important Group of Freshwater Bacterioplankton, by*
515 *Characterization of 35 Isolated Strains. PLoS One 8(3):e58209*
516 *60. Schloss PD, et al. (2009) Introducing mothur: Open-Source, Platform-Independent,*
517 *Community-Supported Software for Describing and Comparing Microbial*
518 *Communities. Appl Environ Microbiol 75(23):7537-7541*

Figure Legends

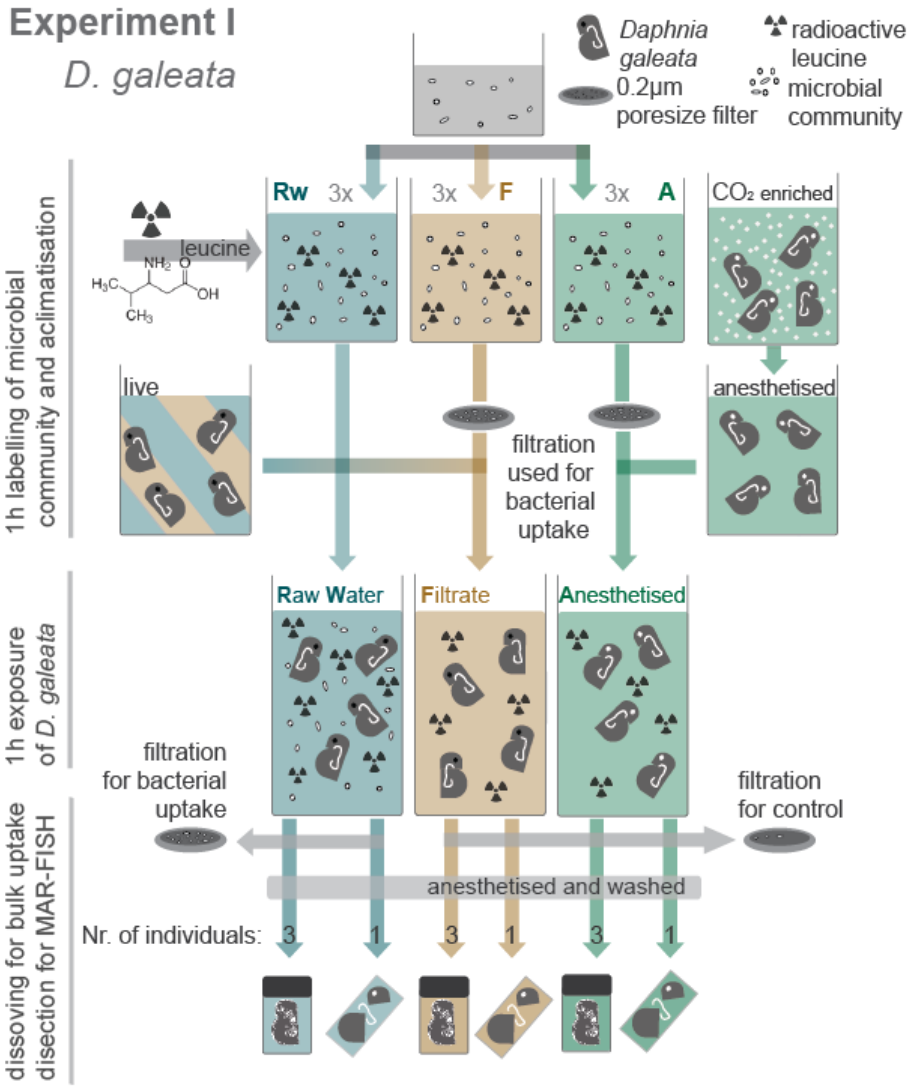

Figure 1: Schematic depiction of the setup of experiment I. For the raw water (Rw) and filtrate (F) treatments active *D. galeata* individuals were incubated for 1 h together with the lake water microbial assemblages (Rw) or in lake water filtrates (F). Both Rw and F treatments had been pre-incubated with tritiated leucine for 1 h before the addition of daphnids. In the anesthetised treatment (A) daphnids were placed in CO_2-enriched mineral water prior to incubation in the lake water filtrates (negative control). Subsequently, daphnids were either dissected for microautoradiography (MAR-FISH, 1 animal), or total leucine uptake per individual was determined by scintillation counting (3 animals). All treatments were done in triplicates.

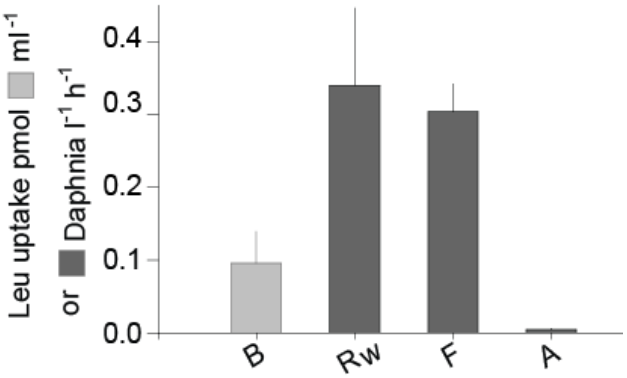

Figure 2: Leucine uptake of the heterotrophic bacterial community (B) after 1h of labelling, by active *Daphnia galeata* after 1h of incubation in the labelled raw water (Rw), and by active (F) and anesthetised (A) *D. galeata* maintained for 1h in tracer containing 0.2 µm prefiltered lake water. Error bars represent the standard deviation of triplicate water samples (B) or the standard errors of measurements from 9 individual daphnids (Rw, F, A).

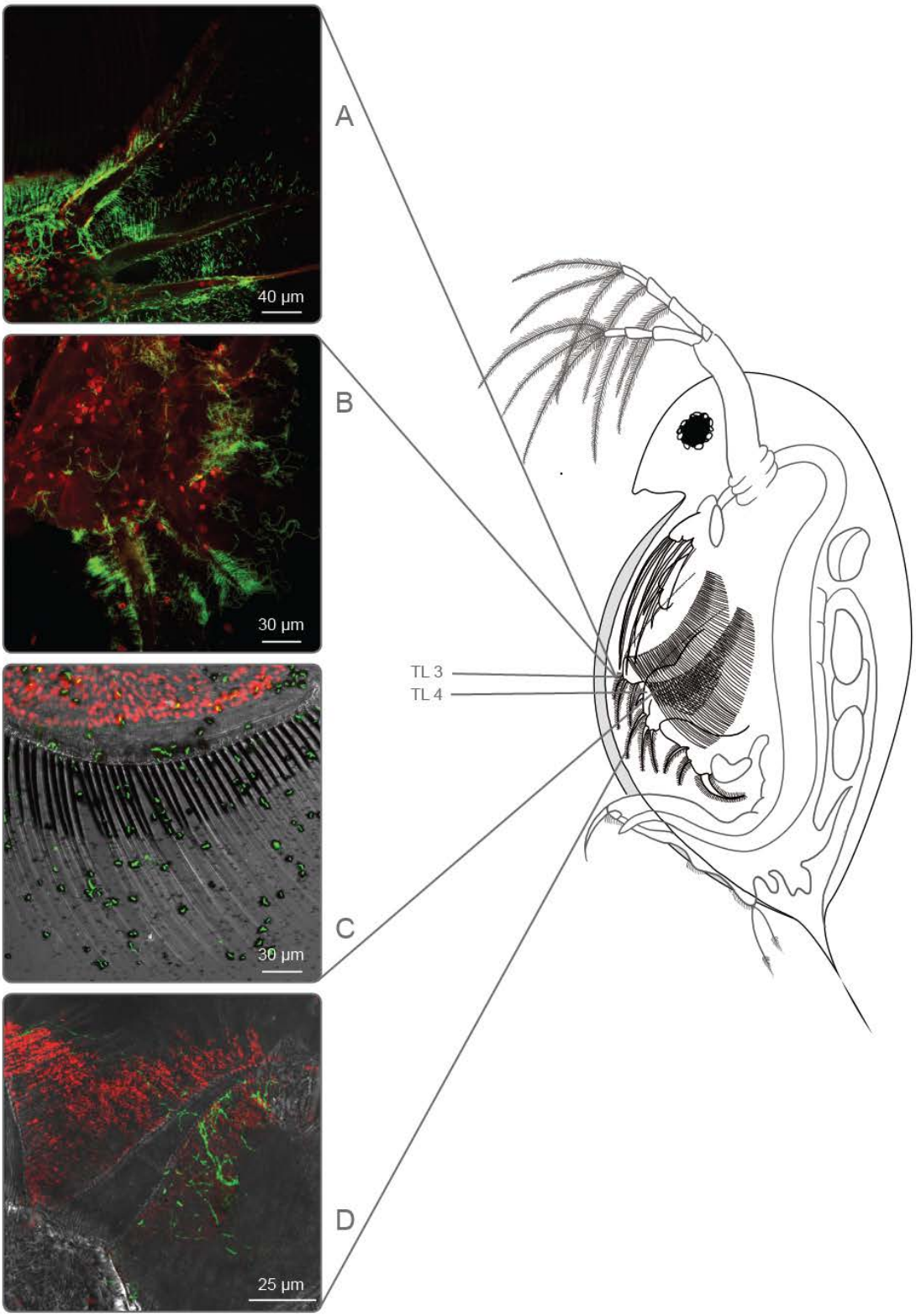

Figure 3: Confocal photomicrographs of *Daphnia spp.* epibionts (left), and localisation of the depicted structures in a schematic drawing of a daphnid (right). Green cells are hybridised with probe R-Bt065, targeting *Lhb* bacteria. Depicted in red are other DNA-containing objects, ie., bacterial cells that are not *Lhb* and nuclei of

Daphnia. Panels A and B: *D. galeata* feeding appendages with hybridised *Lhb cells*. Panel C: *D. galeata* feeding combs with hybridised *Lhb* cells surrounded by black halos from microautoradiography staining that indicates the uptake of tritiated leucine by these bacteria. Panel D: feeding appendage of daphnid from Lake Zurich with hybridised *Lhb* and numerous other bacteria. TL3, TL4: trunk limbs 3 and 4

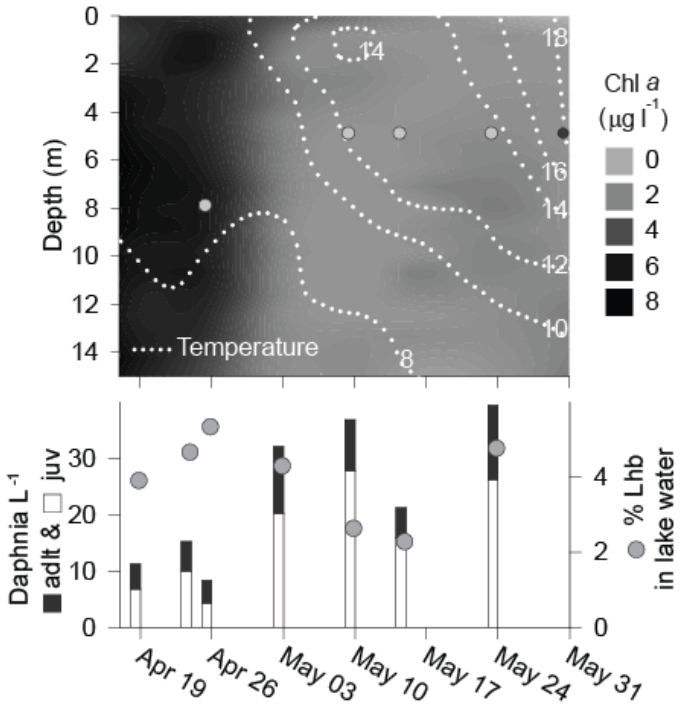

Figure 4: Development of chlorophyll *a*, temperature, and of the populations of *Daphnia sp.* and pelagic *Lhb* bacteria in Lake Zurich from April 17 to May 31 2013. Upper panel: Chlorophyll *a* concentrations and temperature between 0 and 15m depth. Grey circles indicate the dates and depth of samplings for the incubations with radiolabeled tracers, and for DNA extraction to identify Lhb epibionts (last time point). Lower panel: Abundances of juvenile and adult daphnids and proportions of pelagic *Lhb* bacteria of all bacterioplankton cells.

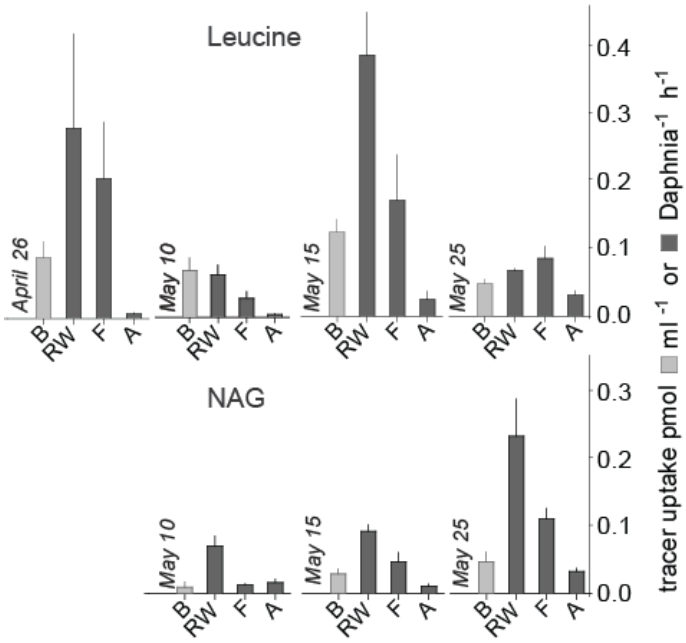

Figure 5: Uptake of tritiated leucine (upper panels) and N-acetyl-glucosamine (NAG, lower panels) by the heterotrophic bacterial community (B) after 1h of incubation, by lake daphnids after 1h of incubation in this labelled raw water (Rw), and by active (F) and anesthetised lake daphnids (A) maintained for 1h in tracer containing 0.2 µm prefiltered lake water. The experimental dates are indicated in each panel. Error bars represent the standard deviation of triplicate water samples (B) or the standard errors of measurements from 9 individual daphnids (Rw, F, A).

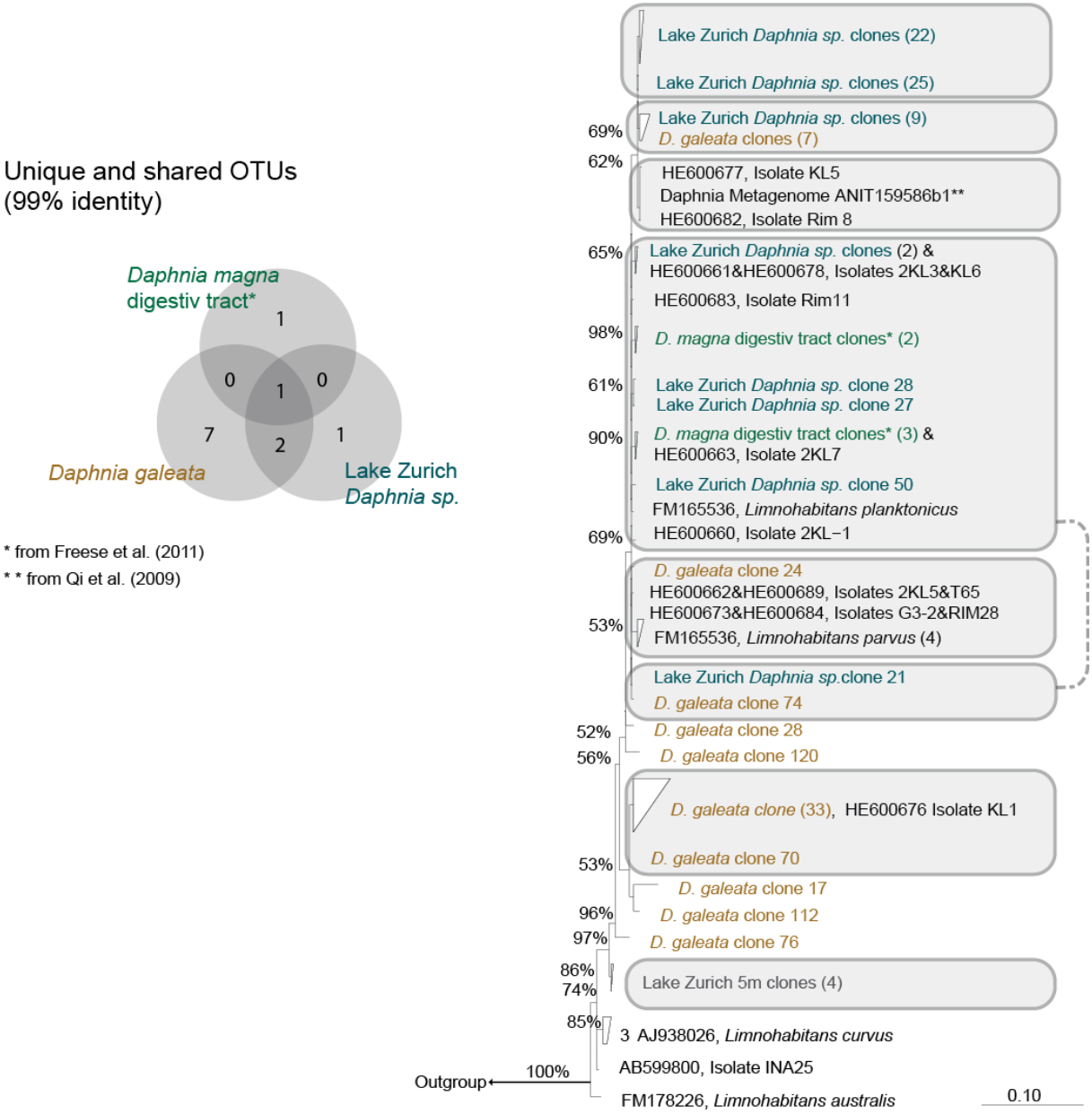

Figure S1: Left panel: Shared and unique operational taxonomic units (OTUs, 99% identity cut-off) of 16S rRNA gene sequences of *Lhb* bacteria from cultured *Daphnia galeata*, from Lake Zurich daphnids, and from cultured *D. magna* (1). Right panel: phylogenetic analysis (Maximum Likelihood method) of *Limnohabitans* spp. including sequences from cultured strains (2). The individual OTUs are depicted as grey boxes, the broken line links sets of sequences from a single OTU. Values in brackets refer to the numbers of sequences in 'collapsed' clusters depicted as wedges. Only bootstrap values >50% (1000 replications) are reported. Scale bar, 10% estimated sequence divergence.

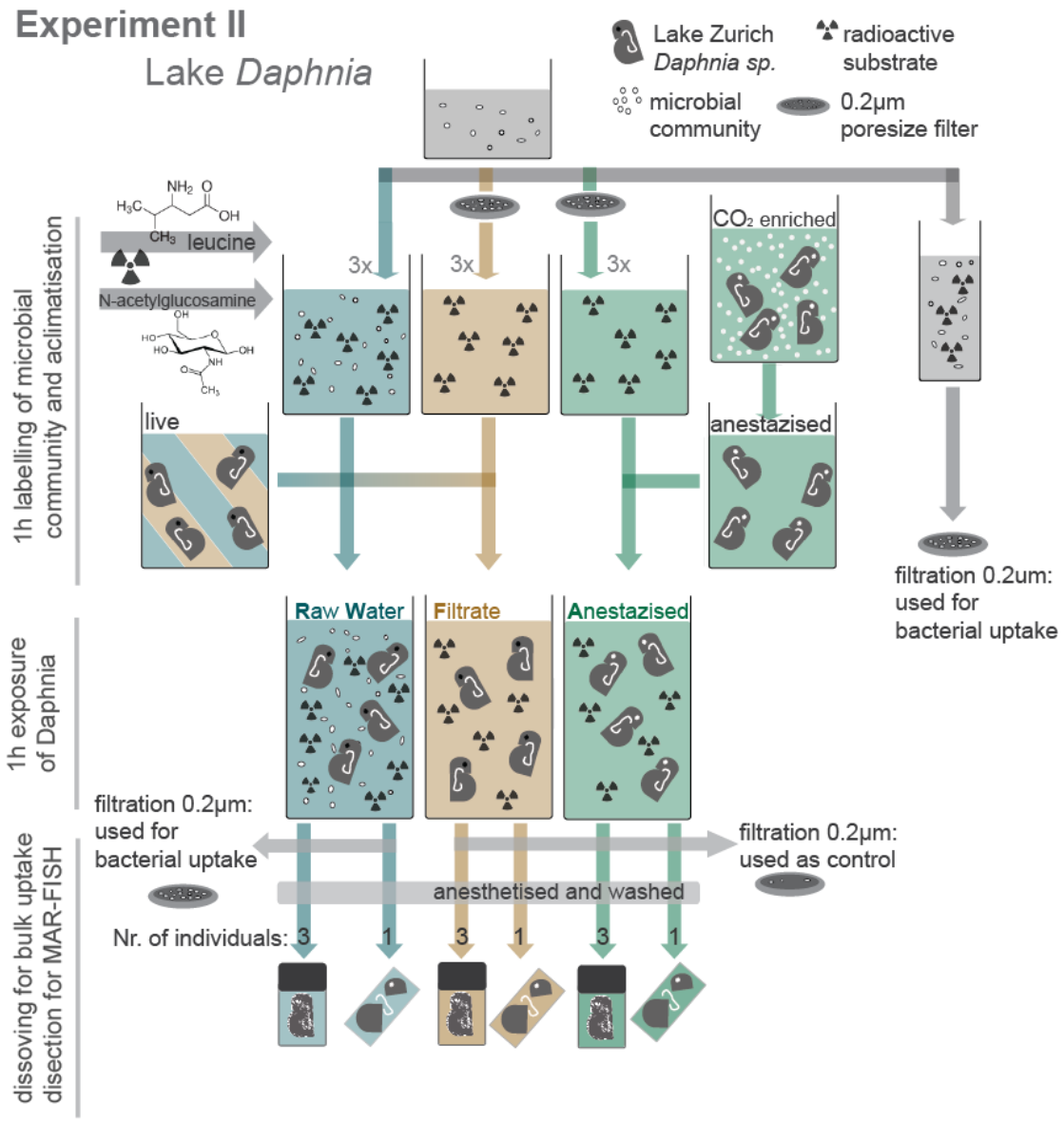

Figure S2: Schematic depiction of the setup of experiment II. For the raw water (Rw) individual *Daphnia* spp. from Lake Zurich were incubated for 1 h together with the lake water microbial assemblages (Rw), that had been pre-incubated with tritiated leucine for 1 h before the addition of daphnids. For the filtrate (F) and the anesthetised treatment (A, negative control) active and CO_2-treated *Daphnia* spp., respectively, were maintained for 1h on lake water filtrates that had been pre-incubated with leucine. Subsequently, daphnids were either dissected for microautoradiography (MAR-FISH, 1 animal), or total leucine uptake per individual was determined by scintillation counting (3 animals). Additionally, lake water samples were incubated with leucine for 1h to determine uptake of the microbial community. All treatments were done in triplicates.

References for Figure S1

1. Freese H, Schink B (2011) Composition and Stability of the Microbial Community inside the Digestive Tract of the Aquatic Crustacean *Daphnia magna*. *Microb Ecol* 62(4):882-894
2. Kasalický V, Jezbera J, Hahn MW, Šimek K (2013) The Diversity of the *Limnohabitans* Genus, an Important Group of Freshwater Bacterioplankton, by Characterization of 35 Isolated Strains. *PLoS One* 8(3):e58209
3. Qi WH, Nong G, Preston JF, Ben-Ami F, Ebert D (2009) Comparative metagenomics of Daphnia symbionts. *BMC Genomics* 10(172):1471-2164

CURRICULUM VITAE

PERSONAL DATA

Full name Ester Maria Eckert
Date of Birth 04.12.1984 | Place of Birth Lisboa, Portugal | Nationality Swiss
Work Address Limnological Station | Seestr. 187 | 8802 Kilchberg | Switzerland
Private Address Birmensdorferstr. 435 | 8055 Zurich | Switzerland
Email ester.eckert@limnol.uzh.ch | ester.eckert@gmail.com

EDUCATION

Oct 2005–Jul 2008	Bachelor in Biology, University of Zurich
Nov 2008–Jan 2010	Master in Microbiology, entitled "Dynamics and ecophysiology of bacteria during a phytoplankton spring bloom in Lake Zurich", supervised by Prof. Jakob Pernthaler, Limnological Station, University of Zurich
Since Mar 2010	PhD-student, supervised by Prof. Jakob Pernthaler, Limnological Station, University of Zurich
Since Okt 2010	Member of the Life Science Zurich Graduate School, Microbiology and Immunology (MIM)

INTERNSHIPS & SELECTION OF EXTERNAL ATTENDED COURSE

Jan –Apr 2005	Internship, communication company Kekst and Company, New York, USA
Jul – Sep 2008	Research internship, focused on the interactions between lake zooplankton and bacteria, working group of Prof. Hans-Peter Grossart, Leibniz Institute for Freshwater Ecology and Inland Fishery, Neuglobsow, Germany
Jul 2011	US-EC Course in Environmental Biotechnology, University of Lausanne, Switzerland
Apr 2012	Insights into DNA barcoding and metabarcoding, ETH Zurich, Switzerland
Nov 2012	4th SitE MICRO course: "Who does what", Neaples, Italy
Jun 2013	Fundamentals and applications of FISH, Porto, Portugal

Publications

Eckert EM, Salcher MM, Posch T, Eugster B & Pernthaler J (2012) Rapid successions affect microbial N-acetyl-glucosamine uptake patterns during a lacustrine spring phytoplankton bloom. Environmental Microbiology 14: 794-806.

Eckert EM, Baumgartner M, Huber IM & Pernthaler J (2013) Grazing resistant freshwater bacteria profit from chitin and cell-wall-derived organic carbon. Environmental Microbiology 15: 2019-2030.

Eckert EM & Pernthaler J (submitted) Bacterial epibionts of Daphnia are a potential route for the efficient transfer of dissolved organic carbon within freshwater food webs.

Callieri C, Coci M, Eckert EM, Salcher MM & Bertoni R (submitted) Archaea and Bacteria in deep lake hypolimnion: in situ dark CO2 uptake and vertical distribution.

Eugster B, Eckert EM, Pomati F, Villiger J, Pernthaler J, Loher E & Posch T (in prep) Diversity and dynamics of pelagic ciliates during a phytoplankton spring bloom.

Conference Contributions

Oral

Feb 2011 Engelberg Switzerland	4th Swiss Microbial Ecology (SME) Meeting	N-Acetyl-Glucosamine incorporation of heterotrophic bacteria during an algal bloom
Aug 2012 Copenhagen Denmark	14th International Symposium on Microbial Ecology (ISME14)	Grazing resistant bacteria profit from organic carbon released during flagellate predation
May 2013 Noordwijk. Netherlands	EuroEEFG: Frontiers in Ecology and Evolutionary Genomics	Feeding on their competitor's remains: Grazing resistant freshwater bacteria profit from organic carbon possibly released through protistan foraging

Poster

Jun 2010 Zurich Switzerland	69th Annual assembly of the Swiss Society for Microbiology (SSM)	Dynamics and ecophysiology of lake bacteria during a phytoplankton spring bloom
Aug 2010 Seattle USA	13th International Symposium on Microbial Ecology (ISME13)	Dynamics and ecophysiology of lake bacteria during a phytoplankton spring bloom
Aug 2011 Rostock Germany	12th Symposium on Aquatic Microbial Ecology (SAME)	Grazing resistant bacteria profit from organic carbon released during flagellate predation

Feb 2013 Ittingen Switzerland	32nd Meeting of the German Society for Protistology (DGP)	Feeding on their competitor's remains: Grazing resistant freshwater bacteria profit from organic carbon possibly released through protistan foraging (Poster award)

Teaching Experience

Mar 2009	Co-supervision of a student group during the block course Bio290; Aquatic Microbial Ecology, University of Zurich
Mar 2010, 2011 & 2012	Planning of a project and supervision of student groups during the block course Bio290; Aquatic Microbial Ecology, University of Zurich
Jun – Jul 2010	Planning and supervision of research internship of Eliane Furrer, University of Zurich
Nov – Dec 2011	Planning and supervision of the bachelor thesis of Iris Huber (Environmental Sciences, ETH Zurich), University of Zurich

Extramural Activities

External expert within the category 'biology and environment' for the national competition of the foundation Schweizer Jugend Forscht (the Swiss youth in science foundation), 2013

External evaluator of abstracts for the 1st EMBO Conference on Aquatic Microbial Ecology: SAME13, 2013, Stresa Italy

Reviewer for Hydrobiologia and Environmental Microbiology

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